ATENT COOPERATION TR[illegible] [illegible]TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 April 2000 (12.04.00)	
International application No. PCT/US99/17595	**Applicant's or agent's file reference** 40750-5002WO
International filing date (day/month/year) 04 August 1999 (04.08.99)	**Priority date** (day/month/year) 04 August 1998 (04.08.98)
Applicant GOUT, Ivan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 March 2000 (03.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Sean Taylor
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

RECEIVED

JAN 18 2000

MORGAN, LEWIS & BOCKIUS LLP

From the INTERNATIONAL SEARCHING AUTHORITY

To:
MORGAN, LEWIS & BOCKIUS LLP
Attn. Adler, R.
1800 M Street, N.W.
Washington, D.C. 20236-5869
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 40750-5002WO	Date of mailing *(day/month/year)* 11/01/2000 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 99/ 17595	International filing date *(day/month/year)* 04/08/1999
Applicant LUDWIG INSTITUTE FOR CANCER RESEARCH et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

Docket 1-19-00 Attorney RGA/INSTR
Case 40750-5002 WO
Due Date 3-11-00
Action Articles 19 Amendment
By PSB Chk Kyng

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Sandra De Jong-van Dam

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATEN. COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
40750-5002WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 17595	04/08/1999	04/08/1998
Applicant		
LUDWIG INSTITUTE FOR CANCER RESEARCH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 17595

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first she t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 10-11, as far as methods in vivo are concerned are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

See FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims 10-11 refer to modulating compounds, and claims 25-26 refer to binding partners of p70 S6K (beta) without giving a true technical characterization. Moreover, no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/17595

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/62 C12N9/12 C12N5/10 C07K16/40
G01N33/50 G01N33/566 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GOUT I. ET AL.: "Molecular cloning and characterization of a novel p70 S6 kinase beta containing a proline-rich region" J. BIOL. CHEM., vol. 273, no. 46, 13 November 1998 (1998-11-13), pages 30061-30064, XP002124654 the whole document ---	1-36
P,X	SAITOH M. ET AL.: "Cloning and characterization of p70(S6 beta) defines a novel family of p70 S6 kinases" BIOCHEM. BIOPHYS. RES. COMMUN., vol. 253, no. 2, 18 December 1998 (1998-12-18), pages 471-476, XP002124655 the whole document ---	1-36

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1999	11/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 24463 A (INCYTE PHARMA INC ;MATHUR PREETE (US); REDDY ROOPA (US); AU YOUNG) 20 May 1999 (1999-05-20) compare nt 180-1450 of seq. ID 10 and nt 101-1370 of seq. ID 1 of present application	1-4
A	WO 98 18935 A (CIBA GEIGY AG ;THOMAS GEORGE (FR); KOZMA SARA (FR)) 7 May 1998 (1998-05-07) abstract claims 1-11	1-36
A	WO 93 19752 A (DANA FARBER CANCER INST INC) 14 October 1993 (1993-10-14) abstract	1-36
A	MUKHOPADHYAY N.K. ET AL.: "An array of insulin-activated, proline-directed serine/threonine kinases phosphorylate the p70 S6 kinase" J. BIOL. CHEM., vol. 267, no. 5, 15 February 1995 (1995-02-15), pages 3325-3335, XP002124656 the whole document	1-36
A	WENG Q.P. ET AL.: "Regulation of the p70 S6 kinase by phosphorylation in vivo" J. BIOL. CHEM., vol. 273, no. 26, 26 June 1998 (1998-06-26), pages 16621-16629, XP002124657 the whole document	1-36
A	ALESSI D.R. ET AL.: "3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro" CURRENT BIOLOGY, vol. 8, 10 December 1997 (1997-12-10), pages 69-81, XP000857264 the whole document	1-36

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAN J -W ET AL: "RAPAMYCIN, WORTMANNIN, AND THE METHYLXANTHINE SQ20006 INACTIVATE P70S6K BY INDUCING DEPHOSPHORYLATION OF THE SAME SUBSET OF SITES" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 270, no. 36, page 21396-21403 XP002057557 ISSN: 0021-9258 the whole document	1-36
A	PEARSON R B ET AL: "THE PRINCIPAL TARGET OF RAPAMYCIN-INDUCED P70S6K INACTIVATION IS A NOVEL PHOSPHORYLATION SITE WITHIN A CONSERVED HYDROPHOBIC DOMAIN" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 14, no. 21, page 5279-5287 XP000561164 ISSN: 0261-4189 the whole document	1-36
A	DENNIS P B ET AL: "THE PRINCIPAL RAPAMYCIN-SENSITIVE P70S6K PHOSPHORYLATION SITES, T-229 AND T-389, ARE DIFFERENTIALLY REGULATED BY RAPAMYCIN-INSENSITIVE KINASE KINASES" MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 16, no. 11, page 6242-6251 XP002057559 ISSN: 0270-7306 the whole document	1-36
A	WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA () 29 January 1998 (1998-01-29) abstract claims 1-9	1-36
A	PROUD C G: "P70 S6 KINASE: AN ENIGMA WITH VARIATIONS" TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 21, no. 5, page 181-185 XP002057556 ISSN: 0968-0004 the whole document	1-36

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US 99/17595

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9924463 A	20-05-1999	US 5932445 A	03-08-1999
		AU 1309599 A	31-05-1999
WO 9818935 A	07-05-1998	AU 5314598 A	22-05-1998
		EP 0942990 A	22-09-1999
WO 9319752 A	14-10-1993	AU 3922493 A	08-11-1993
WO 9803662 A	29-01-1998	AU 4113897 A	10-02-1998
		EP 0915982 A	19-05-1999

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OCT 19 1999

MORGAN, LEWIS & BOCKIUS LLP

PATENT COOPERATION TREATY

PCT

RGA
MST
TFP

From the INTERNATIONAL BUREAU

To:

ADLER, Reid, G.
Morgan, Lewis & Bockius LLP
1800 M Street, N.W.
Washington, DC 20036-5869
ÉTATS-UNIS D'AMÉRIQUE

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 28 September 1999 (28.09.99)	
Applicant's or agent's file reference 40750-5002WO	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/17595	International filing date (day/month/year) 04 August 1999 (04.08.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 04 August 1998 (04.08.98)
Applicant LUDWIG INSTITUTE FOR CANCER RESEARCH et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
04 Augu 1998 (04.08.98)	60/095,268	US	24 Sept 1999 (24.09.99)

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Carlos Naranjo CSN
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/304 (July 1998)

002867940

DOCKETED
By BB Date 10-20-99

PATENT COOPERATION TREATY

PCT

RGA MST TEP FF

From the INTERNATIONAL BUREAU

To:

ADLER, Reid, G.
Morgan, Lewis & Bockius LLP
1800 M Street, N.W.
Washington, DC 20236-5869
ETATS-UNIS D'AMERIQUE

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year) 12 April 2000 (12.04.00)	
Applicant's or agent's file reference 40750-5002WO	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/17595	04 August 1999 (04.08.99)	04 August 1998 (04.08.98)

Applicant
LUDWIG INSTITUTE FOR CANCER RESEARCH et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,JP,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   None

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. (41-22) 740.14.35

Authorized officer:
Sean Taylor
Telephone No. (41-22) 338.83.38

4

PATENT COOPERATION TREATY

REC'D 02 NOV 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
40750-5002WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/17595	04/08/1999	04/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/54		
Applicant LUDWIG INSTITUTE FOR CANCER RESEARCH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 13 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03/03/2000	30.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Surdej, P Telephone No. +49 89 2399 7334

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/17595

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-47 as originally filed

Claims, No.:

1-36 as originally filed

Drawings, sheets:

1/19-19/19 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have b en considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☒ This report has been established as if no priority had been claimed due to the fact that the priority claim has

been found invalid.

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see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 7, 10-11, 25-26.

because:

☒ the said international application, or the said claims Nos. 7, 10-11 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 10-11, 25-26.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2,6-9,17-24,28-36
	No:	Claims	1,3-5,12-16,27
Inventive step (IS)	Yes:	Claims	2,6-9,17-24,28-36
	No:	Claims	1,3-5,12-16,27
Industrial applicability (IA)	Yes:	Claims	1-6,8-9,12-24,27-36
	No:	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cit d

1. Certain published documents (Rule 70.10)

   and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

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The following defects in the form or contents of the international application have been noted:

see separate sheet

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The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

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D3: WO 99 24463 A (INCYTE PHARMA INC ;MATHUR PREETE (US); REDDY ROOPA (US); AU YOUNG) 20 May 1999 (1999-05-20)

D4: WO 98 18935 A (CIBA GEIGY AG ;THOMAS GEORGE (FR); KOZMA SARA (FR)) 7 May 1998 (1998-05-07)

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D6: MUKHOPADHYAY N.K. ET AL.: 'An array of insulin-activated, proline-directed serine/threonine kinases phosphorylate the p70 S6 kinase' J. BIOL. CHEM., vol. 267, no. 5, 15 February 1995 (1995-02-15), pages 3325-3335

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D10: PEARSON R B ET AL: 'THE PRINCIPAL TARGET OF RAPAMYCIN-INDUCED P70S6K INACTIVATION IS A NOVEL PHOSPHORYLATION SITE WITHIN A CONSERVED HYDROPHOBIC DOMAIN' EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 14, no. 21, page 5279-5287

D11: DENNIS P B ET AL: 'THE PRINCIPAL RAPAMYCIN-SENSITIVE

P70S6K PHOSPHORYLATION SITES, T-229 AND T-389, ARE DIFFERENTIALLY REGULATED BY RAPAMYCIN-INSENSITIVE KINASE KINASES' MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 16, no. 11, page 6242-6251

D12: WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA () 29 January 1998 (1998-01-29)

D13: PROUD C G: 'P70 S6 KINASE: AN ENIGMA WITH VARIATIONS' TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 21, no. 5, page 181-185

D14: GROVES ET AL. 'Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini' MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 11, no. 11, page 5541-5550, D14 is not cited in the International Search Report, but it is known to the applicant (see page 1, line 29)

Introduction

The application discloses a new S6 protein kinase, its sequence and structure, methods to identify modulators of its phosphorylation, antibodies against it, fusion proteins containing it, transformed cell with the gene encoding it, methods to identified its substrates and its binding partners, its binding partners, methods to determined its aberrant cellular level, and vectors containing it.

Re Item I

Basis of the report

1. The pages 1-11 of the listing of the sequences 1-8 filed with the international application are taken into account for the establishment of this report.

Re Item II

Priority

2. The application draws priority from a document which has the date 4 August 1998 (P1).
   The generation of activated mutants of p70beta^{S6k} referred to in claim 24 and part

of claim 35, described in the application, is not disclosed in the priority document P1. Therefore, the priority date of P1 cannot be acknowledged and only the filing date of the application will be considered for the subject matter of **claim 24 and part of claim 35**.
Since documents D1-D3 are published before the filing date of the application, they can be cited as prior art for the subject-matter not to be considered entitle to the priority date of P1.
Claims 1-9,12-23, 27-34, part of 35 and 36 seem to be entitled to the priority date of the P1 document.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

3. No opinion is given on the subject-matter of **claims 10-11** and **claims 25-26** since no search report was established on said subject-matter.

4. For the assessment of the present **claims 7, 10-11** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

   Claims 7, 10-11 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item IV

Lack of unity of invention

5. Document D1-D3 are published in the priority interval. The main part of the invention is disclosed in D1. The same protein (p70beta^{S6k}) was also identified in

D2. A protein with a sequence identical to part of p70beta^{S6k} was disclosed in D3. In particular, activated forms were described (see for example Fig. 3 in D1 and Fig. 4 in D2).

6. Therefore, the constitutive activated form of p70beta^{S6k}, which is referred to in claim 24 and part of 35, is not linked by a special technical feature to the subject-matter referred into claims 1-23, 25-34, part of 35 and 36 and they represent two separate inventions. The requisite unity of invention (Rule 13.1 PCT) therefore no longer exists inasmuch as a technical relationship involving a special technical feature in the sense of Rule 13.2 PCT does not exist.

7. Additional lack of unity seems to be present in the subject-matter referred to in claims 12-14, 27. These claims refer to antibodies recognising the protein p70beta^{S6k}.
Such antibodies were already disclosed in the prior art, as stated in the application, since an anti-phosphoserine antibody, commercially available before the priority date, recognises the p70beta^{S6k}.
Therefore, no special technical feature, in the sense of Rule 13.2 PCT, links the different antibodies recognising p70beta^{S6k} and each antibody may represent a separate invention. The claimed subject matter of claims 12-14, 27 seems not so linked as to form a single general inventive concept (Rule 13.1 PCT) and represents a group of separate inventions in regard to the subject-matter referred to in claims 1-11, 15-26, 28-36.

8. The term "stringent condition" used in claims 1 and 3 is not clearly defined in the description. Therefore, the scope of the said claims is not clear (Art. 5 and 6 PCT). As the conditions of hybridization are not defined, e.g. the isolated nucleic acid molecules p70alpha^{S6k}, which are largely disclosed in D4-D14 together with their activities, appear to fall into the scope of the said claims. Since claims 1 and 3 are not new, no special technical feature in the sense of Rule 13.2 PCT links claims 1, 3, 5 and 16, on one hand, and claims 2, 4, 6-15 and 17-36, on the other hand. They seem not so linked as to form a single general inventive concept (Rule 13.1 PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Novelty and inventive step (Art. 33.1-3)

9. Opinion on novelty and inventive step is given with the following proviso:
The term "p70beta^{S6k}" in claims 6-14, 17-31 and 35-36 is understood as referring to the protein with the sequence SEQ ID No.2 which appear to be new.

10. For reasons of clarity (see points 23 and 24): the second part of claim 2 "a protein having one or more conservative amino acid substitution in SEQ ID No.2" and the features "fragment" and "fusion protein" in claims 4, 15 and 17 are not taken into account for the reasoned statement on novelty and inventive step.

11. **Claims 1, 3 and 5** appear not to be new for the reason given in point 8.

12. **Claims 4 and 15** are not new because the proteins p70alpha^{S6k} and p70beta^{S6k} have 70% identity, therefore polypeptide fragments of p70alpha^{S6k}, as defined in the description, fall into the scope of the said claims.

13. **Claim 16** is not new. Said claim refers to cells transformed with nucleic acid molecules referred to in claims 1 and 3 which are not new (see point 8). Such transformed cells are already disclosed, for example, in D7 (page 16622, 2nd column, 4th paragraph), D8 (page 80, 2nd column), D9 (page 21397, 2nd column, 1st paragraph).

14. **Claims 12-14 and 27** are not new for the reasons given in point 7.

15. **Claims 2, 6-9, 17-24, 28-31 and 32-36** refer to an unknown protein having SEQ ID No.2. Therefore, said claims are new. The closest prior art document D14 discloses an isolated nucleic acid molecule having a sequence of a p70 S6 kinase. There are no indications of other member of the p70 S6 kinase family in the prior art and, as stated for example in D12 (page 29, 2nd paragraph) and in D13, it appears that p70^{S6k} is represented by a single gene in mammals. Thus,

said claims appear to involve an inventive step.

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
D3: WO 99/24463	20/05/99	04/11/98	7/11/97

16. The applicants' attention is drawn to the fact that D3 will be considered as prior art in some PCT Contacting States. The EPO, for example, will consider D3 as prejudicial to the novelty of the subject-matter of the present application (Article 54(3) and (4) EPC) insofar as the same Designated Contracting States are concerned.

Re Item VII

Certain defects in the international application

17. The expression "incorporated by reference" (page 1, line 8; page 12, line 15, line 22, line 26; page 47, line 3) seems to imply that other subject matter is incorporated by reference, however the application should be self-explanatory (Art. 5 and 6, Rule 9.1 (iv) and Preliminary Examination Guidelines Ch. II-4.17 PCT).

18. Legibility of Figure 1 is somewhat lacking since the correspondance between identical bases cannot be clearly seen. The bands for p70beta^{s6k} cannot be clearly seen on Fig.4B (Art. 5 PCT).

19. In the legend of Figure 4A, it is not clear what are the different lanes (Art. 5 PCT).

20. It appears that the subject-matter of the last paragraph of page 19 is not related to the subject-matter of the invention (Rule 9.1 iv) and Art. 5 PCT).

21. The vague statement "spirit of the invention" on page 47, line 1 implies that the

subject-matter of the invention may be different from what is defined by the claims, thereby resulting in lack of clarity (Art. 6 PCT) when use to interpret them (see also the PCT Preliminary Examination Guidelines Ch. III-4.3a).

Re Item VIII

Certain observations on the international application

22. The term "conservative" used in claim 2 is defined on page 11, 4th paragraph of the description as referring to "alterations in the amino acid sequence that do not adversely affect the biological functions of the protein". However, it is mentioned in said paragraph that "conservative substitutions may irreversibly activate the protein" and it is not clear whether such changes do not adversely affect the biological function of the protein. Thus, the definition of conservative is not clear (Art. 5 and 6 PCT).

23. The term "fragment" used, for example, in claims 4, 15, 17 is not clear and any fragment seems to fall into the scope of such claims. The same observation applies for the term "fusion protein" (Art. 5 and 6 PCT).

24. The dependence of claim 13 is not clear since the said claim refers to a method in which no antibody are claimed. Thus, the scope of the claim is not clear (Art. 6 PCT).

25. The expression "a first" cellular extract in claims 19 and 21 is not clear since there is no reference to other extracts in the said claims (Art. 6 PCT).

26. In claim 8, it cannot be seen how the modulation of the phosphatase activity by an agent can be determined since the phosphatase seems not to be exposed to the agent. Therefore, the said claim lacks clarity and its scope is not clear (Art. 6 PCT).

27. The term "pcDNA3" employed in claim 34 and which appears to be a registered trade mark has no precise meaning as it is not internationally accepted as a standard descriptive term, thereby rendering the definition of the subject-matter of the said claim unclear (Article 6 PCT).

PATENT COOPERATION TREATY

RGA
MST
TFP
FF

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

POCHE, Thomas F. et al
MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, N.W.
Washington, D.C. 20036-5869
ETATS-UNIS D'AMERIQUE

RECEIVED
NOV 03 2000
MORGAN, LEWIS & BOCKIUS LLP

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	30.10.2000
Applicant's or agent's file reference 40750-5002WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/17595	04/08/1999	04/08/1998

Applicant
LUDWIG INSTITUTE FOR CANCER RESEARCH et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

DOCKETED
By IGF Date 11/9/00

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Emslander, S Tel.+49 89 2399-8718

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
40750-5002WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/17595	04/08/1999	04/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/54		
Applicant LUDWIG INSTITUTE FOR CANCER RESEARCH et al.		

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Date of submission of the demand	Date of completion of this report
03/03/2000	30.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Surdej, P Telephone No. +49 89 2399 7334

Form PCT/IPEA/409 (cover sheet) (January 1994)

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International application No. PCT/US99/17595

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	No:	Claims	1,3-5,12-16,27
Industrial applicability (IA)	Yes:	Claims	1-6,8-9,12-24,27-36
	No:	Claims	

2. Citations and explanations

see separate sheet

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D8: ALESSI D.R. ET AL.: '3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro' CURRENT BIOLOGY, vol. 8, 10 December 1997 (1997-12-10), pages 69-81

D9: HAN J -W ET AL: 'RAPAMYCIN, WORTMANNIN, AND THE METHYLXANTHINE SQ20006 INACTIVATE P70S6K BY INDUCING DEPHOSPHORYLATION OF THE SAME SUBSET OF SITES' JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 270, no. 36, page 21396-21403 ISSN: 0021-9258

D10: PEARSON R B ET AL: 'THE PRINCIPAL TARGET OF RAPAMYCIN-INDUCED P70S6K INACTIVATION IS A NOVEL PHOSPHORYLATION SITE WITHIN A CONSERVED HYDROPHOBIC DOMAIN' EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 14, no. 21, page 5279-5287

D11: DENNIS P B ET AL: 'THE PRINCIPAL RAPAMYCIN-SENSITIVE

P70S6K PHOSPHORYLATION SITES, T-229 AND T-389, ARE DIFFERENTIALLY REGULATED BY RAPAMYCIN-INSENSITIVE KINASE KINASES' MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 16, no. 11, page 6242-6251

D12: WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA () 29 January 1998 (1998-01-29)

D13: PROUD C G: 'P70 S6 KINASE: AN ENIGMA WITH VARIATIONS' TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 21, no. 5, page 181-185

D14: GROVES ET AL. 'Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini' MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 11, no. 11, page 5541-5550, D14 is not cited in the International Search Report, but it is known to the applicant (see page 1, line 29)

Introduction

The application discloses a new S6 protein kinase, its sequence and structure, methods to identify modulators of its phosphorylation, antibodies against it, fusion proteins containing it, transformed cell with the gene encoding it, methods to identified its substrates and its binding partners, its binding partners, methods to determined its aberrant cellular level, and vectors containing it.

Re Item I

Basis of the report

1. The pages 1-11 of the listing of the sequences 1-8 filed with the international application are taken into account for the establishment of this report.

Re Item II

Priority

2. The application draws priority from a document which has the date 4 August 1998 (P1).
   The generation of activated mutants of p70beta^{S6k} referred to in claim 24 and part

of claim 35, described in the application, is not disclosed in the priority document P1. Therefore, the priority date of P1 cannot be acknowledged and only the filing date of the application will be considered for the subject matter of **claim 24 and part of claim 35**.
Since documents D1-D3 are published before the filing date of the application, they can be cited as prior art for the subject-matter not to be considered entitle to the priority date of P1.
Claims 1-9,12-23, 27-34, part of 35 and 36 seem to be entitled to the priority date of the P1 document.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

3. No opinion is given on the subject-matter of **claims 10-11** and **claims 25-26** since no search report was established on said subject-matter.

4. For the assessment of the present **claims 7, 10-11** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

   Claims 7, 10-11 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item IV
Lack of unity of invention

5. Document D1-D3 are published in the priority interval. The main part of the invention is disclosed in D1. The same protein ($p70beta^{S6k}$) was also identified in

D2. A protein with a sequence identical to part of p70beta^{S6k} was disclosed in D3. In particular, activated forms were described (see for example Fig. 3 in D1 and Fig. 4 in D2).

6. Therefore, the constitutive activated form of p70beta^{S6k}, which is referred to in claim 24 and part of 35, is not linked by a special technical feature to the subject-matter referred into claims 1-23, 25-34, part of 35 and 36 and they represent two separate inventions. The requisite unity of invention (Rule 13.1 PCT) therefore no longer exists inasmuch as a technical relationship involving a special technical feature in the sense of Rule 13.2 PCT does not exist.

7. Additional lack of unity seems to be present in the subject-matter referred to in claims 12-14, 27. These claims refer to antibodies recognising the protein p70beta^{S6k}.
Such antibodies were already disclosed in the prior art, as stated in the application, since an anti-phosphoserine antibody, commercially available before the priority date, recognises the p70beta^{S6k}.
Therefore, no special technical feature, in the sense of Rule 13.2 PCT, links the different antibodies recognising p70beta^{S6k} and each antibody may represent a separate invention. The claimed subject matter of claims 12-14, 27 seems not so linked as to form a single general inventive concept (Rule 13.1 PCT) and represents a group of separate inventions in regard to the subject-matter referred to in claims 1-11, 15-26, 28-36.

8. The term "stringent condition" used in claims 1 and 3 is not clearly defined in the description. Therefore, the scope of the said claims is not clear (Art. 5 and 6 PCT). As the conditions of hybridization are not defined, e.g. the isolated nucleic acid molecules p70alpha^{S6k}, which are largely disclosed in D4-D14 together with their activities, appear to fall into the scope of the said claims. Since claims 1 and 3 are not new, no special technical feature in the sense of Rule 13.2 PCT links claims 1, 3, 5 and 16, on one hand, and claims 2, 4, 6-15 and 17-36, on the other hand. They seem not so linked as to form a single general inventive concept (Rule 13.1 PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Novelty and inventive step (Art. 33.1-3)

9. Opinion on novelty and inventive step is given with the following proviso:
The term "p70beta^{S6k}" in claims 6-14, 17-31 and 35-36 is understood as referring to the protein with the sequence SEQ ID No.2 which appear to be new.

10. For reasons of clarity (see points 23 and 24): the second part of claim 2 "a protein having one or more conservative amino acid substitution in SEQ ID No.2" and the features "fragment" and "fusion protein" in claims 4, 15 and 17 are not taken into account for the reasoned statement on novelty and inventive step.

11. **Claims 1, 3 and 5** appear not to be new for the reason given in point 8.

12. **Claims 4 and 15** are not new because the proteins p70alpha^{S6k} and p70beta^{S6k} have 70% identity, therefore polypeptide fragments of p70alpha^{S6k}, as defined in the description, fall into the scope of the said claims.

13. **Claim 16** is not new. Said claim refers to cells transformed with nucleic acid molecules referred to in claims 1 and 3 which are not new (see point 8). Such transformed cells are already disclosed, for example, in D7 (page 16622, 2nd column, 4th paragraph), D8 (page 80, 2nd column), D9 (page 21397, 2nd column, 1st paragraph).

14. **Claims 12-14 and 27** are not new for the reasons given in point 7.

15. **Claims 2, 6-9, 17-24, 28-31 and 32-36** refer to an unknown protein having SEQ ID No.2. Therefore, said claims are new. The closest prior art document D14 discloses an isolated nucleic acid molecule having a sequence of a p70 S6 kinase. There are no indications of other member of the p70 S6 kinase family in the prior art and, as stated for example in D12 (page 29, 2nd paragraph) and in D13, it appears that p70^{S6k} is represented by a single gene in mammals. Thus,

said claims appear to involve an inventive step.

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
D3: WO 99/24463	20/05/99	04/11/98	7/11/97

16. The applicants' attention is drawn to the fact that D3 will be considered as prior art in some PCT Contacting States. The EPO, for example, will consider D3 as prejudicial to the novelty of the subject-matter of the present application (Article 54(3) and (4) EPC) insofar as the same Designated Contracting States are concerned.

Re Item VII

Certain defects in the international application

17. The expression "incorporated by reference" (page 1, line 8; page 12, line 15, line 22, line 26; page 47, line 3) seems to imply that other subject matter is incorporated by reference, however the application should be self-explanatory (Art. 5 and 6, Rule 9.1 (iv) and Preliminary Examination Guidelines Ch. II-4.17 PCT).

18. Legibility of Figure 1 is somewhat lacking since the correspondance between identical bases cannot be clearly seen. The bands for p70beta^{s6k} cannot be clearly seen on Fig.4B (Art. 5 PCT).

19. In the legend of Figure 4A, it is not clear what are the different lanes (Art. 5 PCT).

20. It appears that the subject-matter of the last paragraph of page 19 is not related to the subject-matter of the invention (Rule 9.1 iv) and Art. 5 PCT).

21. The vague statement "spirit of the invention" on page 47, line 1 implies that the

subject-matter of the invention may be different from what is defined by the claims, thereby resulting in lack of clarity (Art. 6 PCT) when use to interpret them (see also the PCT Preliminary Examination Guidelines Ch. III-4.3a).

Re Item VIII

Certain observations on the international application

22. The term "conservative" used in claim 2 is defined on page 11, 4th paragraph of the description as referring to "alterations in the amino acid sequence that do not adversely affect the biological functions of the protein". However, it is mentioned in said paragraph that "conservative substitutions may irreversibly activate the protein" and it is not clear whether such changes do not adversely affect the biological function of the protein. Thus, the definition of conservative is not clear (Art. 5 and 6 PCT).

23. The term "fragment" used, for example, in claims 4, 15, 17 is not clear and any fragment seems to fall into the scope of such claims. The same observation applies for the term "fusion protein" (Art. 5 and 6 PCT).

24. The dependence of claim 13 is not clear since the said claim refers to a method in which no antibody are claimed. Thus, the scope of the claim is not clear (Art. 6 PCT).

25. The expression "a first" cellular extract in claims 19 and 21 is not clear since there is no reference to other extracts in the said claims (Art. 6 PCT).

26. In claim 8, it cannot be seen how the modulation of the phosphatase activity by an agent can be determined since the phosphatase seems not to be exposed to the agent. Therefore, the said claim lacks clarity and its scope is not clear (Art. 6 PCT).

27. The term "pcDNA3" employed in claim 34 and which appears to be a registered trade mark has no precise meaning as it is not internationally accepted as a standard descriptive term, thereby rendering the definition of the subject-matter of the said claim unclear (Article 6 PCT).

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(54) Title: IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL RIBOSOMAL S6 PROTEIN KINASE

(57) Abstract

A novel S6 kinase, p70β^{S6k}, is described, along with methods of making and using p70β^{S6k} and related nucleic acids. The invention also discloses methods of identifying agents which modulate the activity of p70β^{S6k} and/or its ligands.

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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL RIBOSOMAL S6 PROTEIN KINASE

This invention relates to United States Provisional Application Serial No. 60/095,268, filed August 4, 1988, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention relates to a novel S6 kinase ($p70\beta^{Sk6}$), mutant variants thereof, methods of making and using this S6 kinase, and related nucleic acids and antibodies. The invention also relates to binding partners of the S6 kinase, methods of identifying the binding partners and antibodies thereto.

BACKGROUND OF THE INVENTION

The 40S ribosomal protein S6 is a component of the 40S subunit of eukaryotic ribosomes. The ribosomes are part of the cellular machinery responsible for translation of mRNA and protein synthesis. The S6 protein is phosphorylated in response to certain cellular signaling events such as hormone or growth factor induced cellular proliferation. p70 S6 kinase ($p70^{S6k}$) is responsible for S6 phosphorylation and is believed to be the major physiological S6 kinase in mammalian cells (Proud, 1996 Trends Biochem. Sci. 21: 181-185).

1. p70α S6 Kinase

A. Structure and Function

The first p70 S6 kinase identified was the alpha (α) form. The gene encoding the human p70α S6 kinase ($p70^{S6k}$)was isolated in 1991 (Grove *et al.*, 1991 Mol. Cell.

Biol. 11: 5541-5550). Other p70α S6 kinase sequences have been described in *Mus musculus* (GenBank Accession No. SEG_AB015196S, AB015197, and AB015196), *Xenopus laevis* (GenBank Accession No. X66179), and rat (GenBank Accession No. M57428).

Two p70α S6 kinase isoforms were identified: p70α-I (GenBank Accession No. M60724) and p70α-II (GenBank Accession No. M60725). The two p70α S6 kinase isoforms differ only in their amino termini by 23 amino acid residues resulting in a 70 kD protein and a 85 kD protein. The isoforms are referred to in the literature as $p70^{S6k}$/$p85^{S6k}$ or p70α S6 kinase. Both isoforms share similar activity towards ribosomal protein S6 *in vitro* but are expressed in different cells and tissues. The two isoforms are produced by two mRNA products and are not a result of post-translational modifications. They are serine/threonine kinases and are known to act on the substrate KKRNRTLSVA (SEQ ID No. 7) (Pai *et al.*, 1994 Eur. J. Immunol. 24: 2364-8; and Leighton *et al.*, 1995 FEBS Letters 375: 289-93).

The p70α S6 kinase plays an important role in the progression of cells from G1 to S phase of the cell cycle and in the initiation of protein synthesis. Recently, p70α S6 kinase has been demonstrated to regulate the translation of a class of mRNAs containing an oligopyrimidine tract in their 5' untranslated region. This class of mRNAs, termed 5'TOP mRNAs, represent up to 20% of the a cell's total mRNA. Many of the proteins encoded by 5'TOP mRNAs are translational apparatus proteins and cell-cycle progression proteins.

The p70α S6 kinase has four identified interdependent domains: (1) a catalytic domain, (2) a kinase extension domain, (3) a pseudosubstrate autoinhibitory domain, and (4) the N-terminal domain. The catalytic domain is located in the middle of the protein and is followed by the kinase extension domain, which is a unique feature for the PKA family. The pseudosubstrate autoinhibitory domain is also unique for the p70α S6 kinase, not having been observed in any other known kinases. It possesses 5 phosphorylation sites which are responsible for the p70α S6 kinase regulation. The N-terminal domain mediates the sensitivity for rapamycin, which strongly inhibits serum-

induced phosphorylation and activation of the p70α S6 kinase. This domain may also mediate the interaction with a yet unknown phosphatase.

B. Regulators and Cascades

Growth factors, such as insulin, and mitogens are known to activate *in vivo* p70α S6 kinase (Alessi *et al.*, 1998 Curr. Biol. 8: 69-81). Heat shock also activates p70α S6 kinase (Lin *et al.*, 1997 J. Biol. Chem. 272: 31196-31202). Certain drugs have been identified that regulate p70α S6 kinase activity including: rapamycin, wortmannin, Ro31-8220, GF109203X, LY294002, phenylephrine (PE), PD098059, SQ20006, polymerized collagen, forskolin, interleukin-10 (IL-10), demethoxyviridin, phorbol 12-myristate 13-acetate (PMA), A23187, bombesin and antibodies which recognize the p70α S6 kinase (Proud, 1996; Morreale *et al.*, 1997 FEBS Letters 417: 38-42; Kanda *et al.*, 1997 J. Biol. Chem. 272: 23347-23353; Boluyt *et al.*, 1997 Circ. Res. 81: 176-186; Coolican *et al.*, 1997 J. Biol. Chem. 272: 6653-6662; Koyama *et al.*, 1996 Cell 87: 1069-1078; Busca *et al.*, 1996 J. Biol. Chem. 271: 31824-31830; Crawley *et al.*, 1996 J. Biol. Chem. 271: 16357-16362; and Petritsch *et al.*, 1995 Eur. J. Biochem. 230: 431-8). The immunosuppressant rapamycin (Rap) is the most potent inhibitor of p70α S6 kinase described (Pullen *et al.*, 1997 FEBS Letters 410: 78-82).

p70α S6 kinase is an enzyme which lies downstream of phosphoinositide 3-kinases (PI3-kinase). The mechanisms regulating the p70α S6 kinase have not been fully elucidated. PI3-kinase has recently been shown to activate another phosphoinositide-dependent protein kinase, termed PDK-1. So far, only PDK-1 has been shown to phosphorylate p70α S6 kinase *in vivo*, and this phosphorylation is essential for $p70\alpha^{S6k}$ activity towards ribosomal S6 protein. Wortmannin, a fungal inhibitor which down-regulates the p70α S6 kinase, is believed to act by inhibiting PI-3 kinase. In contrast, another fungal inhibitor, rapamycin, inhibits the p70α S6 kinase by another cascade pathway involving the mammalian target of rapamycin (mTOR; also known as RAFT or FRAP) (Proud, 1996; Stewart *et al.*, 1994 BioEssays 16: 809-815). mTOR is a member of the PIK-related family of protein kinases (Pullen *et al.*, 1997).

Additional regulators of the p70α S6 kinase include, but are not limited to protein kinase B (PKB), Cdc42, and Rac. The role of most of these proteins as p70α S6 kinase regulators has yet to be fully elucidated.

SUMMARY OF THE INVENTION

The present invention is based on our discovery of a new gene which encodes a novel S6 kinase ($p70\beta^{S6k}$). The invention includes isolated nucleic acid molecules selected from the group consisting of an isolated nucleic acid molecule that encodes the amino acid sequence of SEQ ID No.2, (*e.g.*, SEQ ID No.1) an isolated nucleic acid molecule that encodes a fragment of SEQ ID No.2, an isolated nucleic acid molecule which hybridizes to the complement of a nucleic acid molecule comprising SEQ ID No.1 under conditions of sufficient stringency to produce a clear signal and an isolated nucleic acid molecule which hybridizes to the complement of a nucleic acid molecule that encodes the amino acid sequence of SEQ ID No.2 under conditions of sufficient stringency to produce a clear signal.

The present invention further includes the nucleic acid molecules operably linked to one or more expression control elements, including vectors comprising the isolated nucleic acid molecules. The invention further includes host cells transformed to contain the nucleic acid molecules of the invention and methods for producing a protein comprising the step of culturing a host cell transformed with the nucleic acid molecule of the invention under conditions in which the protein is expressed.

The invention further provides an isolated polypeptide selected from the group consisting of an isolated polypeptide comprising the amino acid sequence of SEQ ID No.2, an isolated polypeptide comprising a fragment of SEQ ID No.2, an isolated polypeptide comprising conservative amino acid substitutions of SEQ ID No.2 and naturally occurring amino acid sequence variants of SEQ ID No.2.

The invention further provides an isolated antibody that binds to a polypeptide of the invention, including monoclonal and polyclonal antibodies and fragments

thereof.

The invention further provides methods of identifying an agent which modulates the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2 comprising the steps of: exposing cells which express the nucleic acid to the agent; and determining whether the agent modulates expression of said nucleic acid, thereby identifying an agent which modulates the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2.

The invention further provides methods of identifying an agent which modulates at least one activity of a protein comprising the sequence of SEQ ID No.2 comprising the steps of: exposing cells which express the protein to the agent; and determining whether the agent modulates at least on activity of said protein, thereby identifying an agent which modulates at least one activity of a protein comprising the sequence of SEQ ID No.2.

The invention further provides methods of identifying binding partners for a protein comprising the sequence of SEQ ID No.2 or activated variants thereof, comprising for example, the steps of: exposing said protein to a potential binding partner; and determining if the potential binding partner binds to said protein, thereby identifying binding partners for a protein comprising the sequence of SEQ ID No.2. Exposing may be accomplished by expressing the protein in a cell.

The present invention further provides methods of modulating the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2 comprising the step of: administering an effective amount of an agent which modulates the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2. The invention also provides methods of modulating at least one activity of a protein comprising the sequence of SEQ ID No.2 comprising the step of: administering an effective amount of an agent which modulates at least one activity of a protein comprising the sequence of SEQ ID No.2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleic acid sequence of $p70\beta^{S6k}$ (SEQ ID No.1) and comparison with $p70\alpha^{S6k}$ (SEQ ID No. 3). Sequence analysis of cDNA encoding the p70β S6 kinase. p70β cDNA may encode two isoforms with the use of alternative start codon. The longer form may encode a protein of 495 amino acid residues and the shorter form, 482 amino acids (aa). Alternative start codons and a stop codon are highlighted.

Figure 2A-2B. Amino Acid Sequence of $p70\beta^{S6k}$ (SEQ ID No.2) and comparison with $p70\alpha^{S6K}$ (SEQ ID No. 4).

Figure 2A. Alignment of predicted protein sequences corresponding to the $p70\alpha^{S6K}$ and p70β S6 kinases. Identical amino acids are boxed.

Figure 2B. Comparative analysis of regulatory domains and phosphorylation sites between p70α and β S6 kinases.

Figure 3A - 3B. Tissue Specific Expression of $p70\beta^{S6k}$. Northern blot analysis of poly(A)+ RNA from human tissues (Figure 3A) and tumor cell lines (Figure 3B).

Figure 3A. Nylon membrane containing 2 μg of gel-separated, pre-bound poly(A)+ RNA samples from various human tissues was hybridized with cDNA fragments of $p70\alpha^{S6k}$, $p70\beta^{S6k}$ or β-actin labeled by random priming. The upper, middle and lower panels are autoradiographs probed with $p70\beta^{S6k}$, $p70\alpha^{S6k}$ and β-actin, respectively. Each lane contains mRNA prepared from: heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), pancreas (lane 8), spleen (lane 9), thymus (lane 10), prostate (lane 11), testis (lane 12), ovary (lane 13), small intestine (lane 14), mucosal lining of the colon (lane 15), and peripheral blood leukocytes (lane 16).

Figure 3B. Nylon membrane containing 2 μg of mRNA isolated from tumor cell lines was probed with the 3' cDNA fragment from $p70\beta^{S6k}$, which was labeled by random-prime labeling. Specific binding was determined by autoradiography. Promyelocytic leukemia HL-60 (lane 1), HeLa cell S3 (lane 2, chronic myelogenous leukemia K562 (lane 3), lymphoblastic leukemia MOLT-4 (lane 4), Burkitt's

lymphoma Raji (lane 5), colorectal adenocarcinoma SW480 (lane 6), lung carcinoma (lane 7), and melanoma G361 (lane 8).

Figure 4A - 4B. Phosphorylation of the ribosomal protein S6 (Figure 4A) and its C-terminal synthetic peptide (Figure 4B) by $p70\alpha^{S6k}$ and $p70\beta^{S6k}$. Ribosomal S6 protein (purified ribosomal 40S subunits from liver) and synthetic peptides corresponding to the S6 protein C-terminus (*e.g.*, KEAKEKRQEQIARRRLSSLRASTSKSESSQK-long form (SEQ ID No. 5) and RRRLSSLRASTSKSESSQK-(SEQ ID No. 6) short form) were used to measure the activity of the $p70\alpha^{S6k}$ and $p70\beta^{S6k}$. HEK293 cells were transfected with plasmids containing Flag-tag or EE-tag versions of $p70\alpha^{S6k}$ or $p70\beta^{S6k}$. Recombinant proteins were immunoprecipitated with anti-EE or anti-Flag antibodies and an *in vitro* kinase reaction performed in the presence of the ribosomal 40S subunits or synthetic peptides. After SDS-PAGE analysis, phosphorylation of the S6 protein and synthetic peptides was measured by PhosphoImager and expressed in arbitrary units (PI units).

Figure 5A - 5B. Stimulation of $p70\beta^{S6k}$ Activity by insulin, serum and TPA.

Figure 5A. CHO-IR cells were transfected with mock (lane1) or plasmids containing cDNAs of FLAG-tagged p70α-I (lanes 2 and 3) or FLAG-tagged p70β-II (lanes 4 to 7). After serum starvation for 16 hrs, cells were treated with the vehicle (lanes 2 and 4), 10^{-7} M insulin for 10 min (lanes 3 and 5), 15% serum for 10 min (lane 6) or 500 nM TPA for 30 min (lane7). After cell lysis and subsequent immunoprecipitation with anti-FLAG antibodies, immunoprecipitates were subjected to a p70 S6 kinase assay using 40S subunits as substrates. The reaction mixture was separated by SDS-PAGE, transferred onto PVDF membrane. The membrane was analyzed by autoradiography (upper panel) and then immunoblotted with anti-FLAG antibodies (lower panel). A representative of three experiments is shown. ^{32}P incorporation into S6 was quantified by Molecular Dynamics PhosphorImager™ and is expressed in arbitrary units (PI units).

Figure 5B. Stimulation of the p70α^{S6k} and p70β^{S6k} activity towards ribosomal S6 protein by PDGF in PAE-PDGF-R cells. PAE-PDGF-R cells were transfected with EE-tagged p70α^{S6k} and p70β^{S6k} plasmids using lipofectAMINE. After 24 hr, transfected cells were serum-starved for 16 hr and stimulated with 20 ng/ml PDGF BB (Calbiochem) for 20 min. Control cells were treated with the vehicle under the same conditions. After immunoprecipitation with anti-EE antibodies, an *in vitro* kinase reaction was carried out in the presence of 40S subunits, containing the S6 protein. Reaction mixtures were separated by SDS-PAGE and incorporation of ^{32}P into S6 protein was measured by PhosphoImager.

Figure 6A - 6B. Effects of rapamycin and wortmannin on p70β^{S6k} and p70α^{S6k}. Effects of rapamycin and wortmannin on S6 phosphorylation activity of p70α-I or p70β-II. HEK293 cells were transfected with mock cDNA or plasmids containing FLAG-tagged p70α1 or p70β2 isoforms. After 48 h of transfection, cells were treated with vehicle or indicated concentrations of rapamycin or wortmannin for 30 min. After immunoprecipitation with anti-FLAG antibodies, the kinase activity was determined by a p70 S6 kinase assay using 40S subunits as substrates. The proteins of the reaction mixture were separated by SDS-PAGE, transferred onto PVDF membrane and analyzed by autoradiography (Figures 6A and 6B, upper panels). Subsequent immunoblotting with anti-FLAG antibody confirmed the expression of p70α-I (Figure 6A, lower panel) and p70β-II (Figure 6B, lower panel). A representative of three experiments is shown. ^{32}P incorporation into S6 was quantified by PhosphorImager and is expressed in arbitrary units (PI units).

Figure 7. Interaction of the p70β^{S6k} with different GST/SH3 fusion proteins. HEK293 cells were transiently transfected with EE-tag/p70β^{S6k}. Two days later, cells were lysed and the lysates were immunoprecipitated with anti-EE antibodies. GST/SH3 fusion proteins (1.5 μg each) were incubated with anti-EE tag immunoprecipitates. Specific interaction with p70β^{S6k} was measured by anti-GST

immunoblotting. SH3 domains from different signaling and cytoskeletal proteins were expressed in bacteria as GST fusion proteins and purified nearly to homogeneity using glutathione-Sepharose beads. The GST/SH3 fusion proteins used are: GST (lane 1), p80α subunit of the PI3-kinase (lane 2), GAP (lane 3), PLCγ (lane 4), spectrin (lane 5), crk (lane 6), n-grb2 (lane 7), c-grb2 (lane 8), grb2 full (lane 9), csk (lane 10), fgr (lane 11), fyn (lane 12), src (lane 13), ruk a (lane 14), ruk b (lane 15), ruk c (lane 16), p15 (lane 17), profilin (lane 18) and GST/GAP control (lane 19). "↑" indicates instances of binding between $p70\beta^{S6k}$ and a SH3 containing fusion protein.

Figure 8. Immunoprecipitation and Western blot analysis of the p70β-I and p70β-II isoforms transiently over expressed in HEK293 cells with anti-$p70\beta^{S6k}$ antibodies. The lanes are the same for each panel: mock transfected (lane 1), Flag-p70α-I transfected (lane 2), Flag-p70β-I transfected (lane 3), and Flag-p70β-II transfected (lane 4).

Figure 9. A model for the activation of $p70^{S6}$ Kinase. Schematic presentation of the $p70\alpha^{S6k}$ structure, protein-protein interactions, activation levels and phosphorylation state.

Figure 10. $p70\beta^{S6k}$ mutations. Schematic presentation of substitution mutations engineered into $p70\beta^{S6k}$, including a change of Threonine at amino acid 401 to Aspartic acid (T401D).

Figure 11. $p70\beta^{S6k}$ (T401D) activity. Activity of $p70\beta^{S6k}$ (T401D) variant as compared to wt $p70\beta^{S6k}$ under S6 Kinase and autophosphorylation assays.

Figure 12: $p70\alpha^{S6k}$ (T412D) activity. Activity of $p70\alpha^{S6k}$ (T412D) variant as compared to wt $p70\alpha^{S6k}$ under S6 Kinase and autophosphorylation assays.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The terms "p70α", "p70α^{S6k}" and "p70α ^{S6} kinase" are meant to include the two isoforms, p70 and p85, both of which phosphorylate the ribosomal protein S6. By "p70α-1" and "p70α-I" are meant the p85 isoform of the p70α ^{S6} kinase. By "p70α-2" and "p70α-II" are meant the p70 isoform of the p70α ^{S6} kinase.

The terms "p70β", "p70β^{S6k}" and "p70β ^{S6} kinase" include the newly identified S6 kinase and all its isoforms.

I. General Description

The present invention is based in part on identifying a new gene that encodes a novel S6 kinase (p70β^{S6k}). This new gene and the protein that it encodes are members of the family of S6 kinases, of which the p70α-I and -II (also referred to as p70α-1 and p70α-2) isoforms have already been reported.

The protein can serve as a target for agents that can be used to modulate the expression or activity of the protein. For example, agents may be identified which modulate biological processes associated with ribosomal activity.

The present invention is further based on the development of methods for isolating binding partners that bind to the protein or its activated variants. Probes based on the protein are used as capture probes to isolate potential binding partners, such as other proteins. Dominant negative proteins, DNAs encoding these proteins, antibodies to these proteins, peptide fragments of these proteins or mimics of these proteins may be introduced into cells to affect function. Additionally, these proteins provide a novel target for screening of synthetic small molecules and combinatorial or naturally occurring compound libraries to discover novel therapeutics to regulate ribosomal function.

II. Specific Embodiments

A. The Ribosome Associated Protein

The present invention provides isolated protein, allelic variants of the protein, and conservative amino acid substitutions of the protein, including substitutions that activate the protein. As used herein, the protein or polypeptide refers to a protein that has the human amino acid sequence of depicted in SEQ ID No.2. The invention includes naturally occurring allelic variants and proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the same or similar biological functions associated with the disclosed protein.

As used herein, the family of proteins related to the disclosed protein refer to proteins that have been isolated from organisms in addition to humans. The methods used to identify and isolate other members of the family of proteins related to the disclosed protein are described below.

The proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated protein.

The proteins of the present invention further include conservative variants of the proteins herein described. As used herein, a conservative variant refers to alterations in the amino acid sequence that do not adversely affect the biological functions of the protein. A substitution, insertion or deletion is said to adversely affect the protein when the altered sequence prevents or disrupts a biological function associated with the protein. For example, the overall charge, structure or hydrophobic/hydrophilic properties of the protein can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the biological activities of the protein. Conservative substitutions may irreversibly activate the protein.

Ordinarily, the allelic variants, the conservative substitution variants, the members of the protein family, will have an amino acid sequence having at least 71%-about 75% amino acid sequence identity with the human sequence set forth in SEQ ID No.2, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Homology or identity is determined by **BLAST** (**B**asic **L**ocal **A**lignment **S**earch **T**ool) analysis using the algorithm employed by the programs **blastp, blastn, blastx, tblastn** and **tblastx** (Karlin , et al. Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990) and Altschul, S. F. J. Mol. Evol. 36: 290-300(1993), fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al. (Nature Genetics 6: 119-129 (1994)) which is fully incorporated by reference. The search parameters for **histogram, descriptions, alignments, expect** (i.e., the statistical significance threshold for reporting matches against database sequences), **cutoff, matrix** and **filter** are at the default settings. The default scoring matrix used by **blastp, blastx, tblastn**, and **tblastx** is the **BLOSUM62** matrix (Henikoff, et al. Proc. Natl. Acad. Sci. USA 89: 10915-10919 (1992), fully incorporated by reference). For **blastn**, the scoring matrix is set by the ratios of **M** (i.e., the reward score for a pair of matching residues) to **N** USA 87: 2264-2268 (1990) and (i.e., the penalty score for mismatching residues), wherein the default values for **M** and

N are 5 and -4, respectively.

Thus, the proteins of the present invention include molecules having the amino acid sequence disclosed in SEQ ID No.2; fragments thereof having a consecutive sequence of at least about 3, 5, 10 or 15 amino acid residues of the disclosed protein; amino acid sequence variants of such sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, the disclosed sequence; amino acid sequence variants of the disclosed sequence, or their fragments as defined above, that have been substituted by another residue. Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding proteins of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the family of proteins; and derivatives wherein the protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope). Proteins of the invention may include fusion proteins comprising any of the foregoing.

As described below, members of the family of proteins can be used: 1) to identify agents which modulate at least one activity of the protein, including agents which may modulate phosphorylation mediated by the protein; 2) in methods of identifying binding partners for the protein, 3) as an antigen to raise polyclonal or monoclonal antibodies, and 4) as a therapeutic agent.

B. Nucleic Acid Molecules

The present invention further provides nucleic acid molecules that encode the protein having SEQ ID No.2 and the related proteins herein described, preferably in isolated form. As used herein, "nucleic acid" is defined as RNA or DNA that encodes a peptide as defined above, or is complementary to nucleic acid sequence encoding such peptides, or hybridizes to such nucleic acid and remains stably bound to it under

appropriate stringency conditions, or encodes a polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85%, with the peptide sequences. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such hybridizing or complementary nucleic acids, however, are defined further as being novel and nonobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to nucleic acid encoding a protein according to the present invention.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl, 0.0015 M sodium titrate, 0.1% SDS at 50°C; or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid.

The present invention further provides fragments of the encoding nucleic acid molecule. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the protein, the fragment will need to be large enough to encode the

functional region(s) of the protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the encoding nucleic acid molecules of the present invention (i.e., synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding proteins of the invention can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, (J. Am. Chem. Soc. 103: 3185-3191, 1981) or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can employ any of the art known labels to obtain a labeled encoding nucleic acid molecule.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

C. Isolation of Other Related Nucleic Acid Molecules

As described above, the identification of the human nucleic acid molecule having SEQ ID No.1 allows a skilled artisan to isolate nucleic acid molecules that encode other members of the $p70\beta^{S6k}$ family in addition to the human sequence herein described. Further, the presently disclosed nucleic acid molecules allow a skilled artisan to isolate

nucleic acid molecules that encode other members of the $p70\beta^{S6k}$ family of proteins in addition to the disclosed protein having SEQ ID No.2.

Essentially, a skilled artisan can readily use the amino acid sequence of SEQ ID No.2 to generate antibody probes to screen expression libraries prepared from appropriate cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as *λgtII* library, to obtain the appropriate coding sequence for other members of the protein family. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme.

Alternatively, a portion of the coding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the protein family from any mammalian organism. Oligomers containing approximately 18-20 or 21 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively clone an encoding nucleic acid molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other encoding nucleic acid molecules.

D. rDNA molecules Containing a Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a coding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, (1989). In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the protein family encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the structural gene included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a coding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or bacteriophage promoter capable of directing the expression (transcription and translation) of the coding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form a rDNA molecules the contains a coding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene (Southern *et al.*, J. Mol. Anal. Genet. 1: 327-341, 1982). Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

E. Host Cells Containing an Exogenously Supplied Coding Nucleic Acid Molecule

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a protein of the invention are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), and the like eukaryotic

tissue culture cell lines.

Any prokaryotic host can be used to express a rDNA molecule encoding a protein of the invention. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, Proc. Natl. Acad. Sci. USA 69: 2110, 1972; and Maniatis *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, Virol. 52: 456, 1973; Wigler *et al.*, Proc. Natl. Acad. Sci. USA 76: 1373-76, 1979.

Successfully transformed cells, i.e., cells that contain a rDNA molecule of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, J. Mol. Biol. 98:503, 1975, or Berent *et al.*, Biotech. 3: 208, 1985 or the proteins produced from the cell assayed via an immunological method.

Recombinant $p70\beta^{S6k}$ DNA can also be utilized to analyze the function of coding and non-coding sequences. For example, the 5' untranslated region of the $p70\beta^{S6k}$ clone contains a GA repeat (nucleotides 1-66 of $P70\beta^{S6k}$), that may modulate the initiation of translation of its mRNA. This sequence can be utilized in an affinity matrix system to purify proteins obtained from cell lysates that associate with the $p70\beta^{S6k}$ GA sequence. Synthetic oligonucleotides would be coupled to the beads and probed with the lysates, as is commonly known in the art. Associated proteins could then be separated using, for example, a two dimensional SDS-PAGE system. Proteins thus isolated could be further

identified using mass spectroscopy or protein sequencing.

F. Production of Recombinant Proteins using a rDNA Molecule

The present invention further provides methods for producing a protein of the invention using nucleic acid molecules herein described. In general terms, the production of a recombinant form of a protein typically involves the following steps:

First, a nucleic acid molecule is obtained that encodes a protein of the invention, such as the nucleic acid molecule depicted in SEQ ID No. 1, or particularly for the $p70\beta^{S6k}$ nucleotides encoding the proline rich domain or the amino terminus of $p70\beta^{S6k}$. The coding sequence is directly suitable for expression in any host, as it is not interrupted by introns. The sequence can be transfected into host cells such as eukaryotic cells or prokaryotic cells. Eukaryotic hosts include mammalian cells (*e.g.*, HEK293 cells, CHO cells and PAE-PDGF-R cells) as well as insect cells such as Sf9 cells using recombinant baculovirus. Alternatively, fragments encoding only portion of $p70\beta^{S6k}$ can be expressed alone or in the form of a fusion protein. For example, the C-terminal fragment of $p70\beta^{S6k}$ containing the proline-rich domain, was expressed in bacteria as a GST- or His-tag fusion protein. These fusion proteins were then purified and used to generate polyclonal antibodies.

The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the protein open reading frame. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the recombinant protein. Optionally the recombinant protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with the nucleic acid molecules of the invention to produce recombinant protein.

G. In Vitro Methods to Identify Binding Partners

Another embodiment of the present invention provides methods for use in isolating and identifying binding partners of proteins of the invention In detail, a protein of the invention is mixed with a potential binding partner or an extract or fraction of a cell under conditions that allow the association of potential binding partners with the protein of the invention. After mixing, peptides, polypeptides, proteins or other molecules that have become associated with a protein of the invention are separated from the mixture. The binding partner that bound to the protein of the invention can then be removed and further analyzed. To identify and isolate a binding partner, the entire protein, for instance the entire disclosed protein of SEQ ID No.2 can be used. Alternatively, a fragment of the protein can be used.

As used herein, a cellular extract refers to a preparation or fraction which is made from a lysed or disrupted cell.

A variety of methods can be used to obtain cell extracts. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

Once an extract of a cell is prepared, the extract is mixed with the protein of the invention under conditions in which association of the protein with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that

closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract used, can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to a protein of the invention can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

To aid in separating associated binding partner pairs from the mixed extract, the protein of the invention can be immobilized on a solid support. For example, the protein can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the protein to a solid support aids in separating peptide/binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins.

Alternatively, the nucleic acid molecules of the invention can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the nucleic acid molecules herein described.

One preferred *in vitro* binding assay for $p70\beta^{S6k}$ would comprise a mixture of a polypeptide comprising at least the kinase domain of $p70\beta^{S6k}$ and one or more candidate binding targets or substrates. After incubating the mixture under appropriate conditions, one would determine whether $p70\beta^{S6k}$ or a polypeptide fragment thereof containing the kinase region either bound with the candidate substrate or phosphorylated the candidate substrate. For cell-free binding assays, one of the

components usually comprises or is coupled to a label. The label may provide for direct detection, such as radioactivity, luminescence, optical or electron density, *etc.*, or indirect detection such as an epitope tag, an enzyme, *etc.* A variety of methods may be employed to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and the label thereafter detected.

H. Methods to Identify Agents that Modulate the Expression a Nucleic Acid Encoding the S6 Kinase Protein.

Another embodiment of the present invention provides methods for identifying agents that modulate the expression of a nucleic acid encoding a protein of the invention such as a protein having the amino acid sequence of SEQ ID No.2. Such assays may utilize any available means of monitoring for changes in the expression level of the nucleic acids of the invention. As used herein, an agent is said to modulate the expression of a nucleic acid of the invention, for instance a nucleic acid encoding the protein having the sequence of SEQ ID No.2, if it is capable of up- or down-regulating expression of the nucleic acid in a cell.

In one assay format, cell lines that contain reporter gene fusions between the open reading frame defined by $p70\beta^{S6k}$ nucleotides 77-1,564 or 116-1,564 of SEQ ID No.1 and any assayable fusion partner may be prepared. Numerous assayable fusion partners are known and readily available including the firefly luciferase gene and the gene encoding chloramphenicol acetyltransferase (Alam *et al.*, 1990 Anal. Biochem. 188: 245-254). Cell lines containing the reporter gene fusions are then exposed to the agent to be tested under appropriate conditions and time. Differential expression of the reporter gene between samples exposed to the agent and control samples identifies agents which modulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2.

Additional assay formats may be used to monitor the ability of the agent to

modulate the expression of a nucleic acid encoding a protein of the invention such as the protein having SEQ ID No.2. For instance, mRNA expression may be monitored directly by hybridization to the nucleic acids of the invention. Cell lines are exposed to the agent to be tested under appropriate conditions and time and total RNA or mRNA is isolated by standard procedures such those disclosed in Sambrook *et al.* (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed. Cold Spring Harbor Laboratory Press, 1989).

Probes to detect differences in RNA expression levels between cells exposed to the agent and control cells may be prepared from the nucleic acids of the invention. It is preferable, but not necessary, to design probes which hybridize only with target nucleic acids under conditions of high stringency. Only highly complementary nucleic acid hybrids form under conditions of high stringency. Accordingly, the stringency of the assay conditions determines the amount of complementarity which should exist between two nucleic acid strands in order to form a hybrid. Stringency should be chosen to maximize the difference in stability between the probe:target hybrid and potential probe:non-target hybrids.

Probes may be designed from the nucleic acids of the invention through methods known in the art. For instance, the G+C content of the probe and the probe length can affect probe binding to its target sequence. Methods to optimize probe specificity are commonly available in Sambrook *et al.* (1989) or Ausubel *et al.* (CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Co., NY, 1995).

Hybridization conditions are modified using known methods, such as those described by Sambrook *et al.* (1989) and Ausubel *et al.* (1995) as required for each probe. Hybridization of total cellular RNA or RNA enriched for polyA RNA can be accomplished in any available format. For instance, total cellular RNA or RNA enriched for polyA RNA can be affixed to a solid support and the solid support exposed to at least one probe comprising at least one, or part of one of the sequences of the invention under conditions in which the probe will specifically hybridize. Alternatively, nucleic acid fragments comprising at least one, or part of one of the

sequences of the invention can be affixed to a solid support, such as a porous glass wafer. The glass wafer can then be exposed to total cellular RNA or polyA RNA from a sample under conditions in which the affixed sequences will specifically hybridize. Such glass wafers and hybridization methods are widely available, for example, those disclosed by Beattie (WO 95/11755). By examining for the ability of a given probe to specifically hybridize to an RNA sample from an untreated cell population and from a cell population exposed to the agent, agents which up or down regulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2 are identified.

I. Cell-Based Methods to Identify Binding Partners and Agents that Modulate at Least One Activity of the S6 Kinase Protein and Related Antibodies.

Another embodiment of the present invention provides methods for identifying agents that modulate at least one activity of a protein of the invention such as the protein having the amino acid sequence of SEQ ID No.2. Such methods or assays may utilize any means of monitoring or detecting the desired activity.

In one format, the relative amounts of a protein of the invention between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein in the different cell populations. Cell lines or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysates may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysates are then analyzed with the probe.

For example, N- and C- terminal fragments of $p70\beta^{S6k}$ can be expressed in bacteria and used to search for proteins which bind to these fragments. Fusion proteins, such as His-tag or GST fusion to the N- or C-terminal regions of $p70\beta^{S6k}$ can be prepared for use as a $p70\beta^{S6k}$ fragment substrate. These fusion proteins can be coupled to Talon or Glutathione-Sepharose beads and then probed with cell lysates. Prior to

lysis, the cells may be treated with rapamycin or other drugs which may modulate $p70\beta^{S6k}$ or proteins that interact with $p70\beta^{S6k}$. Lysate proteins binding to the fusion proteins can be resolved by SDS-PAGE, isolated and identified by protein sequencing or mass spectroscopy, as is known in the art. It is likely that signaling molecules containing one or more SH3 domains may bind directly to the C-terminal region of $p70\beta^{S6k}$. The N-terminal domain may have a $p70\beta^{S6k}$-specific phosphatase as a binding partner.

Antibody probes are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptides, polypeptides or proteins of the invention, such as $p70\beta^{S6k}$, variants and isolated binding partners, if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a Cys residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

Anti-peptide antibodies can be generated using synthetic peptides corresponding to, for example, the carboxy terminal 15 amino acids $p70\beta^{S6k}$. Synthetic peptides can be as small as 1-3 amino acids in length, but are preferably at least 4 or more amino acid residues long. The peptides are coupled to KLH using standard methods and can be immunized into animals such as rabbits. Polyclonal anti-$p70\beta^{S6k}$ peptide antibodies can then be purified, for example using Actigel beads containing the covalently bound peptide.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten. polypeptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid. Of particular interest, are monoclonal antibodies which recognize the proline-rich domain of $p70\beta^{S6k}$.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as Fav, $_{sc}$FV, Fab, Fab', or $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin. Antibodies may preferably also be human, humanized or chimeric variants of the foregoing. Such antibodies can be less immunogenic when administered to a subject. Methods of producing humanized or chimeric antibodies are well known in the art. The antibodies contemplated also include different isotypes and isotype subclasses (*e.g.*, IgG_1, IgG_2, IgM, to name a few). These antibodies can be prepared by raising them in vertebrates, in hybridoma cell lines or other cell lines, or by recombinant means. For references on how to prepare these antibodies, see E. Harlow and D. Lane, ANTIBODIES: A LABORATORY MANUAL (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1988); Kohler and Milstein, (1976) E. J. Immunol. 6:511; Queen *et al.* U.S. Patent NO. 5,585,089; and Riechmann *et al.*, Nature 332:323 (1988).

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of receptor

can also be produced in the context of chimeras with multiple species origin.

In an alternative format, a specific activity of a protein of the invention may be assayed, such as the ability of the protein to phosphorylate a substrate such as polypeptides of the S6 protein. For example, $p70\beta^{S6k}$ has been demonstrated to phosphorylate the S6 protein and a synthetic peptide, RRLSSLRASTSKSESSQK (SEQ ID No. 8). The sequence comprising the synthetic peptide is located in the C-terminus of the S6 protein and is known to contain the five phosphorylation sites targeted by $p70\alpha^{S6k}$. Cell lines or populations are exposed under appropriate conditions to the agent to be tested. Agents which modulate the kinase activity of the protein of the invention are identified by assaying the kinase activity of the protein from the exposed cell line or population and a control, unexposed cell line or population, thereby identifying agents which modulate the kinase activity of the protein. Polypeptides of the S6 protein, such as the above examples, are useful positive controls in identifying additional $p70\beta^{S6k}$ substrates.

Kinase assays to measure the ability of the agent to modulate the kinase activity of a protein of the invention are widely available such as the assays disclosed by Mishima *et al.* (1996) J. Biochem. 119: 906-913) and Michnoff *et al.* (1986) J. Biol. Chem. 261: 8320-8326. Alternative assay formats include actin-myosin motility assays such as those disclosed by Kohama *et al.* (1996) TIPS 17: 284-287 or Warrick *et al.* (1987) Ann. Rev. Cell. Biol. 3: 379-421.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. As described

in the Examples, there are proposed binding sites for ATP/GTP and calmodulin as well as cAMP/cGMP kinase sites, TyrP sites and Ser/Thr kinase (catalytic) sites in the protein having SEQ ID No.2. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up these sites. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the ATP or calmodulin binding sites or domains.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of proteins of the invention. Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies.

J. Uses for Agents that Modulate at Least One Activity of the S6 Kinase Protein.

As provided in the Examples, the proteins and nucleic acids of the invention, such as the protein having the amino acid sequence of SEQ ID No.2, are involved in ribosomal function. Agents that modulate or down-regulate the expression of the protein or agents such as agonists or antagonists of at least one activity of the protein may be used to modulate biological and pathologic processes associated with the protein's function and activity.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by a protein of the invention. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological process mediated by a protein of the invention may include binding of substrates such as ATP, GTP or calmodulin or phosphorylation of a substrate, such as the S6 protein.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, expression or up-regulation of expression of a protein of the invention may be associated with certain diseases. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, a disease may be prevented or disease progression modulated by the administration of agents which reduce or modulate in some way the expression or at least one activity of a protein of the invention.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which modulate expression or at least one activity of a protein of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise about 0.1 to

100 μg/kg body weight. The preferred dosages comprise about 0.1 to 10 μg/kg body weight. The most preferred dosages comprise about 0.1 to 1 μg/kg body weight. In tissue culture, optimal dosage ranges for drugs such as wortmannin and rapamycin range from about 500 pM to about 1000 nM. Less optimum ranges include about 10 pM to about 10 mg.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be coadministered along with other compounds typically prescribed for these

conditions according to generally accepted medical practice. The compounds of this invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

EXAMPLE 1

Amino Acid Sequence of $p70\beta^{S6k}$ and comparison with $p70\alpha^{S6k}$

Materials and Methods. Restriction enzymes and DNA modification enzymes were obtained from standard commercial sources and used according to manufacturer's recommendations. Oligonucleotides which were used for sequencing of $p70\beta^{S6k}$ and various PCR fragments were synthesized by Genosys or Japan Bioservice, Inc. The pcDNA1 and pcDNA3 mammalian expression vectors were from Invitrogen. The pGEX-4T vector, glutathione-Sepharose-4B and HiTrapQ columns were purchased from Pharmacia. cDNA of rat p70α-I was a gift from Dr. Joseph Avruch (Diabetes Unit, Massachusetts General Hospital). Rapamycin and PDGF BB were purchased form Calbiochem. Wortmannin was purchased from Sigma.

Cell cultures and Antibodies. A porcine aortic endothelial cell line (PAE-PDGF-R), stably expressing the human PDGF-β receptor, was maintained in HAM's F12 medium containing 10% fetal calf serum (FCS). CHO cells stably overexpressing human insulin receptors (CHO-IR cells) and HEK293 cells were maintained and cultured as described earlier (Hara *et al.*, 1998 J. Biol. Chem. 273: 14484-14494) in HAM's F12 medium or Dulbecco's modified Eagles minimal essential medium (DMEM) supplemented with 10% FCS, respectively. Anti-FLAG monoclonal M2

antibody was purchased from Eastman Kodak Corp. Anti-phosphopeptide antibodies against proline-directed site Ser434 of p70α-I were purchased from New England Biolabs. Polyclonal antibodies raised against the C-terminal 104 amino acids fragment of $p70\alpha^{S6k}$ were from Dr. Joseph Avruch. A GST fusion protein containing amino acids 443-495 of $p70\beta^{S6k}$ (p70βC Ab) was used to raise polyclonal antibodies specific for $p70\beta^{S6k}$. Immunoreactive sera were affinity-purified on an Affigel matrix containing the GST/$p70\beta^{S6k}$-terminal fusion protein.

Fractionation of cell extracts. HEK293 cells were starved in DMEM medium for 16 h and then treated with 15% FCS for 10 min or 200 nM rapamycin for 30 min. After treatment, cells were lysed in ice-cold buffer A (20mM Tris/HCl pH 7.5, 20 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM β-glycerophosphate, 1 mM-DTT, 1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin) and the lysates centrifuged at 4°C for 20 min at 10,000xg. Supernatants were filtered through a 0.45 μM filter and then loaded onto a HighTrapQ Sepharose column (1.0 ml column volume) equilibrated in Buffer A. The column was washed extensively in buffer A and bound proteins eluted with a linear gradient of NaCl (20 - 500 mM). Aliquots of eluted proteins were subjected to SDS- PAGE and immunoblotted with anti-phosphopeptide antibodies directed against proline-directed site Ser-434 of $p70\alpha^{S6k}$ or anti-peptide antibodies against the carboxyl terminal end of $p70\alpha^{S6k}$.

Construction and screening of a HEK293 Uni-ZAP library and DNA sequencing analysis. Total RNA was isolated from HEK293 cells as described (Hara *et al.*, 1998; Chomczynski *et al.*, 1987 Analytical Biochem. 162: 156-159), and poly(A)+ mRNA was selected by using Dynabeads mRNA purification kit (Dynal). An oligo(dT)-primed library was constructed in UNI-ZAP XR vector from 5 mg of HEK293 mRNA, using the Uni-ZAP cDNA synthesis kit (Stratagene). Packaging into phages was carried out by using Gigapack III Gold Packaging extracts (Stratagene). The cDNA encoding full length $p70\beta^{S6k}$ kinase was isolated by screening $1x10^6$ primary phages from HEK293 Uni-ZAP library with a ^{32}P-labeled 0.65 Kb *EcoRI/NotI* fragment derived from the EST clone GenBank Accession No. AA410355 (Hillier *et al.*,

published on GenBank, 1997). Positive clones were confirmed, isolated in second-round screening and rescued as Bluescript plasmids by *in vivo* excision (Stratagene). PCR amplification and restriction mapping were used for primary characterization of isolated clones. Sequencing analysis of selected clones was performed on an Applied Biosystem 373A DNA automatic sequencer (PE Applied Biosystems).

Results. The $p70\alpha^{S6k}$ is activated by multiple phosphorylation within the pseudosubstrate and catalytic domains in response to extracellular stimuli, including serum, growth factors and hormones. Phosphospecific antibodies directed against phosphorylated sites of $p70\alpha^{S6k}$ have been recently developed: Phospho-$p70\alpha^{S6k}$ (Ser434) and Phospho-$p70\alpha^{S6k}$ (Thr444/Ser447). Both antibodies were shown to recognize specifically phosphorylated forms of $p70\alpha^{S6k}$, and this recognition was found to be sensitive to rapamycin. To compare the phosphorylation state and chromatographic behavior of $p70\alpha^{S6k}$ from cells stimulated with serum and treated with or without rapamycin, HEK293 cells were starved in DMEM medium for 16 h and then stimulated with 15% serum for 10 min prior treatment with or without 200 nM of rapamycin for 30 min. Cell extracts were fractionated using HighTrapQ Sepharose columns. Proteins were resolved on SDS-PAGE and immunoblotted with anti-phosphopeptide antibodies Ser434 and S444/T447 or antibodies specific for $p70\alpha^{S6k}$. In total lysates of serum stimulated HEK293 cells, this antibody recognizes specifically phosphorylated versions of $p70\alpha^{S6k}$ isoforms (p70 and p85). However, when cell lysates were fractionated and separated on SDS-PAGE, several additional bands appeared on the immunoblot together with the p70 and p85 isoforms of $p70\alpha^{S6k}$, including p190, p110, p90 and p60 (not shown). It is important to note that the recognition of these proteins by phosphospecific S434 antibodies was sensitive to rapamycin, indicating the specificity for the phosphorylated epitope. As expected, the anti-peptide antibodies specific for the $p70\alpha^{S6k}$ recognized 85-kDa and 70-kDa bands, which correspond to p70α-I and p70α-II, respectively. S434 is located in the autoinhibitory region of the $p70\alpha^{S6k}$, which is highly conserved among different species of $p70\alpha^{S6k}$, including Drosophila (Stewart *et al.*, 1996 Proc. Natl Acad. Sci. USA 93:

10791-10796; Watson *et al.*, 1996 Proc. Natl Acad. Sci. USA 93: 13694-13698). As phosphorylation of the S434 site is sensitive to rapamycin, it is possible to speculate that p190, p110, p90 and/or p60 may represent novel rapamycin-sensitive $p70\alpha^{S6k}$-related kinases.

Molecular cloning of novel $p70\alpha^{S6k}$. Peptide sequences which were used for raising anti-phosphopeptide antibodies S434 of p70α-I were taken to search the expressed sequence tag (EST) databases. This search generated hundreds of EST clones that showed high degree of homology to the query sequence. Extensive analysis of these clones allowed us to isolate several clones which were highly homologous to the 434 peptide, but did not match to cDNA clones from EMBL or Swissprot databases. Further characterization of these sequences indicated that two nearly identical clones (GenBank Accession Nos. AA284234 and AA410355) exhibited strong homology to the kinase extension domain of the protein kinase A (PKA) family of serine/threonine (S/T) kinases. Additionally, the homology in the kinase extension domain extended into the putative autoinhibitory domain, which is unique for the $p70\alpha^{S6k}$ (less than 75% identity between $p70\alpha^{S6k}$ and the unknown potential S6 kinase). However, the homology with $p70\alpha^{S6k}$ dropped significantly downstream of the kinase extension and autoinhibitory domains, suggesting that these clones encoded a novel kinase of this family. Based on these data, we decided to further characterize these clones. Both EST clones were obtained from the UK HGMP Resource Center. Restriction mapping indicated that the GenBank Accession Nos. AA284234 and AA410355 clones contain short inserts of 0.6 kB and 0.65 kB respectively. Sequence analysis showed that these clones are identical to each other in an overlapping region and may encode a partial open reading frame (ORF), which shows very strong homology to the kinase extension and autoinhibitory domains of the $p70\alpha^{S6k}$. These ESTs did not contain a full gene nor was there a protein coding sequence previously identified in these ESTs. Furthermore, four of the five proline-directed Ser/Thr phosphorylation sites located in an autoinhibitory pseudo-substrate domain of $p70\alpha^{S6k}$ were conserved in the clones of $p70\beta^{S6k}$. Immediately after the autoinhibitory pseudo-substrate domain, the homology

between p70α^{S6k} and p70β^{S6k} was very low (12% identity). Taking in account these findings, we proceeded to clone the full length cDNA clone encoding this potentially novel kinase.

Since several rapamycin-sensitive bands were found in the HEK293 cells with the use of anti-Ser434 phosphospecific antibodies, a library from this cell line was created. We screened 10^6 primary clones from the Uni-Zap/HEK293 library with a full length insert from EST clone AA410355 and isolated 12 positive clones. Sequence analysis of rescued plasmids allowed us to identify one clone, which contained an open reading frame of 495 amino acids (Figure 2A). The C-terminus of this clone was found to be identical to the sequence of the EST clone AA410355, which was used for screening.

By analogy to p70α^{S6k}, the novel cDNA, encoding p70β^{S6k}, could potentially encode two isoforms as a result of alternative start codons. If this is the case, the shorter isoform may utilize an ATG codon which is 13 amino acids (aa) downstream of the first methionine and may encode a protein of 482 amino acids. Two potential isoforms were termed p70β-I (495 aa long) and p70β-II (482 aa long). The presence of additional 13 aa at the N-terminus of p70β-I isoform may determine its subcellular localization in the nucleus due to the presence of a putative nuclear localization sequence (RGRRARG, amino acid numbers 3-9 of SEQ ID No. 2). The overall structure of p70β^{S6k} is similar to that of p70α^{S6k}. p70α^{S6k} and p70β^{S6k} share 70% identity and 85% similarity on protein level. The p70β^{S6k} kinase consists of the amino-terminal non-catalytic region, a catalytic domain, a kinase extension and a carboxyl-terminal non-catalytic tail, whose amino acid identity corresponds to domains of p70α^{S6k} is 40%, 83%, 80%, and 47%, respectively (Figure 2B). The strong argument that this clone encodes a novel p70 S6 kinase is the presence of the autoinhibitory pseudosubstrate domain, which is not present in any other known kinases.

p70α^{S6k} undergoes a multi-site phosphorylation in response to stimulation by insulin or mitogens. Such multiple phosphorylation sites are also well conserved in p70β^{S6k} (Figure 2B). It also contains 3 sets of phosphorylation sites, similar to p70α^{S6k}:

(i) a set of Ser/Thr-Pro motifs clustered in an autoinhibitory pseudosubstrate domain (Ser423, Ser430, Ser436, Ser441 in $p70\beta^{S6k}$ correspond to Ser 434, 441, and 447, Ser 452 in $p70\alpha^{S6k}$; (ii) a second set includes Ser383 and Thr401 which is located in the kinase extension domain and corresponds to Ser394 and Thr412 in $p70\alpha^{S6k}$; (iii) a third set consists of Thr251 which resides in the activation T-loop of kinase domain and corresponds to Thr252 in $p70\alpha^{S6k}$. The greatest difference between the $p70\alpha^{S6k}$ and the $p70\beta^{S6k}$ sequences are in the amino-terminal non-catalytic region (40% identity and 60% similarity) and the carboxyl-terminal non-catalytic tail (47% identity and 66% similarity). $p70\beta^{S6k}$ also contains proline-rich sequences at the C-terminus, that may mediate the interaction with SH3-domain-containing molecules.

EXAMPLE 2

Tissue Specific Expression of $p70\beta^{S6k}$

Materials and Methods. Northern blot analysis was performed using commercial nylon membranes pre-bound with 2 μg of gel-separated poly(A)+ RNA samples obtained from various human tissues or tumor cell lines from Clontech. The following probes were used for the detection of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ messages: (i) a 476-base pair (bp) *HindIII* fragment spanning 56 bp of 3' end coding region and 420 bp of 3' non-coding region of the human $p70\alpha^{S6k}$ (EST clone, AA425599), (ii) a 650-bp fragment spanning 518 bp upstream of the stop codon and about 130 bp of non-coding region of the human $p70\beta^{S6k}$ (EST clone, AA410355). Human β-actin cDNA probe was used as a negative control (Clontech). These probes were labeled by Multiprime DNA labeling system (Amersham) and separated from unincorporated [γ^{32}P]dCTP by Nuctrap push columns (Stratagene). Northern blots were pre-hybridized with ExpressHyb solution and hybridized with labeled probes according to manufacturer's recommendations. After extensive washing with 2X SSC, 0.05% sodium dodecyl sulfate (SDS) at room temperature, and twice with 0.1X SSC, 0.1% SDS at 50°C, the localization of bound probes on membranes was identified by autoradiography or with the use of the PhosphoImager.

Results. In order to compare expression patterns of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ in human tissues and cell lines, 3' prime coding and non-coding regions, which exhibit low level of homology between both the α and β S6 kinases, were used as probes. Northern blot analysis using poly(A)+ RNA isolated from human tissues revealed a single 2.2 kb transcript for $p70\beta^{S6k}$, while $p70\alpha^{S6k}$ probe specifically hybridized to 3.4 kb and 7.4 kb transcripts (Figure 3A). The expression pattern of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ transcripts is remarkably similar, showing ubiquitous expression in all tissues. Highest expression levels were found in spleen, skeletal muscle and peripheral blood leukocytes, whereas brain, lung and kidney showed the lowest expression of transcripts for both S6 kinases. The only significant difference on the level of mRNA expression between $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ was found in liver. The expression of $p70\beta^{S6k}$ mRNA in liver is 2-3 times higher than that of $p70\alpha^{S6k}$. Liver possesses a high concentration of $p70\alpha^{S6k}$ and was originally used by several groups to purify $p70\alpha^{S6k}$ for biochemical studies and protein sequencing analysis (Banerjee *et al.*, 1990 Proc. Natl Acad. Sci. USA 87: 8550-8554; Kozma *et al.*, 1990 Proc. Natl Acad. Sci. USA 87: 7365-7369).

We also analyzed the expression of $p70\beta^{S6k}$ mRNA in tumor cell lines using the same probe as for the analysis of tissue distribution. A single transcript of the same size as in human tissues, 2.2 kb, was found to be highly expressed in HeLa and K562 cells, but was barely detectable in HL-60, MOLT-4 and melanoma G361 cell lines (Figure 3B).

EXAMPLE 3

Phosphorylation of the ribosomal protein S6 and its C-terminal synthetic peptide by $p70\alpha^{S6k}$ and $p70\beta^{S6k}$

Materials and Methods. Expression of GST/$p70\beta^{S6k}$ fusion protein in bacteria. A PCR-based strategy was used to make a bacterial expression plasmid for GST/$p70\beta^{S6k}$ fusion protein. A cDNA fragment encoding 443-495 amino acids of $p70\beta^{S6k}$ was amplified by PCR and cloned into the pGEX-4T expression vector (Pharmacia). This construct was transformed into *E. coli* XL1-Blue competent cells

(Stratagene) and the expression of the GST/p70β^{S6k} fusion protein was induced by isopropyl β-D-thiogalactopyranoside (IPTG). The GST/p70β^{S6k} fusion protein was purified by using glutathione-Sepharose-4B beads according to manufacturer's recommendation (Pharmacia). After SDS-PAGE analysis, affinity purified fusion proteins were dialyzed against 20 mM (Tris pH 7.4), 150 mM NaCl, 50% Glycerol and stored at -20°C. This preparation of the GST/p70β^{S6k} C-terminal fragment was used for the production of polyclonal antibodies specific for p70β^{S6k}.

Construction of mammalian expression plasmids. The full length coding sequence, corresponding to the p70β-I (I-495 amino acids) was amplified by PCR using human cDNA clone N53 isolated from HEK293 library as a template and a panel of specific oligonucleotides. Amplified constructs were digested with appropriate enzymes, gel purified and cloned into the pcDNA1 vector in-frame with N-terminal FLAG epitope.

Amino-terminal EE-tagged p70α-II and p70β-II constructs were created by a PCR-based cloning strategy. This was achieved by using specific oligonucleotides containing EE-tag sequence and appropriate restriction sites. The cDNA encoding full length human p70β^{S6k} (clone 53) and rat p70α^{S6k} were used as templates. The resulting PCR fragments were digested with restriction enzymes, gel purified and cloned into the pcDNA3 expression vector. The construction of the pMT2 FLAG p70α-I was described previously (Hara *et al.*, 1998). All constructs generated by a PCR-based approach were verified by sequencing. A Qiagen plasmid Midi kit was used to purify plasmid DNAs for transient transfections. The introduction of the FLAG-tag and the EE-tag sequences at the N-terminus of p70α^{S6k} and p70β^{S6k} allows the study of recombinant proteins via the use of specific antibodies.

Results. To test if the isolated p70β^{S6k} cDNA would encode a functional kinase capable of phosphorylating ribosomal protein S6, a cDNA fragment encoding a short version of p70β^{S6k} (p70β-II) was subcloned into mammalian expression vector in frame with EE-tag or Flag-tag epitopes. These constructs were transfected into HEK293 cells using lipofectAMINE under conditions recommended by the manufacturer. The

expression of recombinant p70β^{S6k} was analyzed by immunoprecipatation or western blotting with EE-tag or Flag-tag antibodies. Both constructs express the protein of approximately 60kDa. The expression level of p70β^{S6k} was comparable to that of p70α-I and p70α-II isoforms, when expressed in HEK293 cells.

Anti-p70β^{S6k} polyclonal antibodies were generated using synthetic peptides corresponding to the carboxy terminal 15 amino acids of p70β^{S6k}. These peptides were coupled to KLH and then injected into rabbits using standard procedures. Immune sera was purified using Affigel beads containing covalently cross-linked carboxy terminal peptide.

To determine whether the putative p70β^{S6k} was indeed a novel ribosomal protein S6 kinase, the recombinant p70β^{S6k} was expressed in HEK293 cells, immunoprecipitated with anti-EE-tag antibodies and an *in vitro* kinase reaction performed in the presence of purified 40S ribosomal subunit. As shown in Figure 4A, p70β^{S6k} (p70β-II isoform) phosphorylates the S6 protein *in vitro* nearly as efficiently as p70α^{S6k}. We also tested the ability of p70β^{S6k} (p70β-II isoform) to phosphorylate a synthetic peptide representing C-terminus of S6 protein, which contains all sites known to be phosphorylated by p70α^{S6k}. Figure 4B demonstrates that p70β^{S6k} (p70β-II isoform) also phosphorylates this peptide but with slightly lower efficiency than observed with p70α^{S6k}. Therefore, p70β^{S6k} (p70β-II isoform) may not phosphorylate the same sites of the ribosomal protein S6 as does p70α^{S6k}.

The intrinsic activity of the p70β^{S6k} is significantly lower than p70α^{S6k}. The absence of one phosphorylation site in the autoinhibitory domain of p70β^{S6k} may be responsible for this decrease in intrinsic activity.

EXAMPLE 4

Stimulation of p70β^{S6k} Activity by insulin, serum and TPA

Materials and Methods. HEK293 cells or CHO-IR cells were transfected with plasmids containing p70α^{S6k} and p70β^{S6k} sequences using lipofectAMINE under conditions recommended by the manufacturer (Gibco-BRL). Two days later,

transfected cells were frozen in liquid nitrogen and stored until lysis. After cell extraction, the lysates were subjected to immunoprecipitation and/or immunoblot analysis. If cells were to be stimulated, they were starved in medium without FCS for 16 h and then stimulated with 10^{-7} M insulin for 10 min, 15% FCS for 10 min, 500 nM TPA for 30 min or vehicle alone. PAE-PDGF-R cells were transfected with appropriate plasmids using lipofectAMINE under conditions recommended by the manufacturer. After 24 h transfected cells were serum-starved for 16 h and stimulated with 20 ng/ml PDGF BB (Calbiochem) for 20 min. Control cells were treated with the vehicle under the same conditions.

Results. The effect of various extracellular stimuli on the $p70\beta^{S6k}$ activity was studied in different cell lines, transiently transfected with Flag-tagged or EE-tagged versions of $p70\beta^{S6k}$. The activation of $p70\beta^{S6k}$ (p70β-II isoform) by insulin was analyzed in CHO-IR cells, which stably over expresses the insulin receptor. As shown in Figure 5A, treatment of CHO-IR cells with insulin induces $p70\beta^{S6k}$ (p70β-II isoform) activity towards ribosomal S6 protein by 2.8 fold. In the same cell line and under the same conditions, the activity of $p70\alpha^{S6k}$ was activated 3.5 fold with insulin treatment (Figure 5A). Almost equal amounts of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ (p70β-II isoform) were expressed in cells and used in an *in vitro* kinase assay after immunoprecipitation. In addition, both serum and TPA also stimulated $p70\beta^{S6k}$ (p70β-II isoform) phosphorylation of the ribosomal protein S6 (Figure 5A).

Using PAE-PDGF-R cells, the activation of the $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ (p70β-II isoform) by PDGF was examined. This cell line stably over expresses the PDGF receptor, and the activation of $p70\alpha^{S6k}$ in response to PDGF was shown to be very efficient (Figure 5B). We found that PDGF stimulation of these cells leads to a rapid activation of recombinant $p70\beta^{S6k}$ (p70β-II isoform).

These data indicate that $p70\beta^{S6k}$ is activated by a number of extracellular stimuli in a very similar way as $p70\alpha^{S6k}$. However, activation in the PAE-PDGF-R cells was 30 fold for $p70\beta^{S6k}$ and only 3.4 fold for $p70\alpha^{S6k}$.

EXAMPLE 5

Effects of rapamycin and wortmannin on p70β^{S6k} and p70α^{S6k}

Materials and Methods. Treatment of transfected cells with rapamycin or wortmannin was performed as follows: 48 h after transfection, the cells were treated with various concentrations of rapamycin or wortmannin for 30 min.

Results. These data in Examples 4 and 5 indicate that p70β^{S6k} is activated by a number of extracellular stimuli in a similar fashion to p70α^{S6k}. The two fungal inhibitors, wortmannin and rapamycin, specifically inhibit activation of p70α^{S6k} via PI3-kinase- and mTOR-dependent pathways respectively. Thus, the effects of both inhibitors on p70β^{S6k} activity were examined. After p70α-I and p70β-II were transiently expressed in HEK293 cells, cells were maintained in DMEM containing 10% FCS and then treated with various concentrations of rapamycin or wortmannin. We found that the activity of p70α^{S6k} and p70β^{S6k} were inhibited by rapamycin and wortmannin in a dose dependent manner (Figures 6A and B, upper panel). However, it appears that p70β^{S6k} is less sensitive to rapamycin and wortmannin, when compared with p70α^{S6k}. This difference is more obvious at lower concentration of inhibitors. In the presence of 20 nM rapamycin the inhibition of the p70α^{S6k} is 92%, while only 46% for p70β^{S6k}. Addition of 100 nM of wortmannin inhibits 86% of p70α^{S6k} activity and 62% of p70β^{S6k} activity. The inhibition of p70β^{S6k} by rapamycin and wortmannin is lower than that observed for p70α^{S6k} indicating different mechanisms of regulation exist for p70β^{S6k}.

EXAMPLE 6

Interaction of the p70β^{S6k} with different GST/SH3 fusion proteins

Materials and Methods. The EE-tag/p70β^{S6k} was transiently over expressed in HEK293 cells as described above. Transfected cells were lysed in buffer A (50 mM-Tris/HCl pH = 8.0, 1% NP-40, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 20 mM sodium β-glycerophosphate, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 2 μg/ml aprotinin) two days after transfection. After

centrifugation at 12,000 rpm for 20 min, the supernatants were incubated for 2 hrs with Protein A beads pre-coupled with anti-EE antibodies. Beads were washed with lysis buffer before different GST/SH3 fusion proteins (1.5 mg each) were added to separate immunoprecipitation reactions. Two hours later, beads were washed extensively in lysis buffer, bound proteins separated on the SDS-PAGE and transferred to the PVDF membrane. Specific interaction of the GST/SH3 domains with pre-bound EE-tag/p70β^{S6k} was assessed by immunoblotting with the anti-GST antibodies.

Results. The C-terminus of the p70β^{S6k} contains proline-rich sequences which are not present in p70α^{S6k}. Src-homology region 3 domains (SH3 domain) are present in many signaling and cytoskeletal molecules and interact specifically with proline-rich sequences which form left-handed helixes. Sequence analysis of the proline-rich region in p70β^{S6k} indicates the presence of several putative SH3 domain binding motifs. Therefore, the ability of p70β^{S6k} (p70β-II isoform) to interact with a panel of SH3 domains was examined. In this experiment, the EE-tag/p70β^{S6k} was transiently over expressed in HEK293 cells and immunoprecipitated with anti-EE antibodies coupled to Protein G Sepharose. The resulting immunoprecipitates were incubated with different GST/SH3 domain fusion proteins. After extensive washing, specific interaction between p70β^{S6k} and SH3 domains was analyzed by SDS-PAGE and immunoblotting with anti-GST antibodies. As shown in Figure 7, several SH3 domains, including those of GAP, Src, Fgr exhibited specific interaction towards p70β^{S6k}.

EXAMPLE 7

Immunoprecipitation and Western blot analysis of transiently expressed p70β-I and p70β-II

Materials and Methods. Anti-p70β^{S6k} polyclonal antibodies were generated using a synthetic peptide corresponding to the C-terminal tail of p70β^{S6k}. The peptide was coupled to KLH, and rabbits were immunized using standard procedures. Immune sera harvested obtained from the immunized rabbits was purified using affinity chromatography on Affigel beads containing covalently cross-linked C-terminal

peptides of $p70\beta^{S6k}$.

HEK293 cells were transfected with pcDNA1 along, pcDNA1/Flag-p70α-I, pcDNA1/Flag-p70β-I, or pcDNA1/Flag-p70β-II. Two days after transfection with one of these plasmids, cell lysates were prepared. Proteins were immunoprecipitated using the $p70\beta^{S6k}$ C-terminal affinity purified polyclonal antibodies or anti-Flag monoclonal antibodies. Immunoprecipitates were resolved on SDS PAGE and proteins transferred to PVDF membranes. The PVDF membranes were immunoblotted using anti-Flag monoclonal antibodies or $p70\beta^{S6k}$ C-terminal antibodies as indicated in Figure 8.

Results. The expression of both $p70\beta^{S6k}$ isoforms was analyzed in HEK293 cells using anti-$p70\beta^{S6k}$ C-terminus specific polyclonal antibodies. Both $p70\beta^{S6k}$ isoforms were found to be specifically immunoprecipitated with anti-$p70\beta^{S6k}$ antibodies, but not p70α-I, as confirmed by anti-Flag and anti-$p70\beta^{S6k}$ immunoblotting. It was found that the p70β-I isoform which encodes a 495 amino acid protein, is translated into a protein which migrates in a SDS-PAGE gel at approximately 70 kD. The p70β-II isoform, which is a truncated form of p70β-I lacking 13 amino acids at the amino terminus of p70β-II, migrates in a SDS-PAGE gel at approximately 60 kD.

EXAMPLE 8

Generation of activated variants of $p70\beta^{S6k}$ (T401D) and $p70\alpha^{S6k}$ (T412D).

Materials and Method. Activated variants of $p70\beta^{S6k}$ and $p70\alpha^{S6k}$ were made by site-directed mutagenesis. Oligonucleotide primers, specific to the site to be mutated and complimentary to opposite strands of $p70\beta^{S6k}$ and $p70\alpha^{S6k}$ sequences were generated as recommended by manufacturer (Stratagene). Site-directed mutagenesis was carried out using pcDNA3/ Glu-tag-$p70\beta^{S6k}$ and pcDNA3/Glu-tag- $p70\alpha^{S6k}$ expression vector/plasmids as templates, according to the recommended protocol (Stratagene). All mutations were verified by DNA sequencing. Expression of mutated forms of both kinases was analyzed by immunobloting with anti-Glu-tag antibodies. The activity of normal and mutated forms of both kinases was measured by in vitro S6 kinase assay. 40S ribosomal subunit was used as a substrate in this reaction.

Results. Studies on p70α^{S6k} demonstrate that this kinase is activated by multiple phosphorylations in response to growth factors or hormones (Fig. 9). A number of kinases that can phosphorylate p70α^{S6k} *in vitro* and *in vivo* have been identified, including cdc2, MAPK, SAPK, p38, TOR and PDK1. However, very little is known about the process of dephosphorylation, which is essential for the inactivation of the kinase.

We have generated an activated form of p70β^{S6k} by substituting putative phosphorylation site Thr 401 with Aspartic acid (Asp) ("p70β^{S6k} (T402D)") as shown in Fig 10. Transfection studies in HEK293 cells and S6 kinase assays indicated that p70β^{S6k} (T401D) mutant is in an activated state in unstimulated cells, when compared with wild type kinase (3 times higher activity, as shown in Fig 11). We have also created an activated version of p70α^{S6k} (T412D), which showed a greater state of activation (18 fold activation, Fig. 12).

It will be apparent to the skilled artisan that activated variants of both kinases such as disclosed above can be used in the search for binding partners. Binding partners or molecules, such as phosphatases, are considered to form tighter and more stable complexes with such irreversibly activated kinases.

EXAMPLE 9

Identification of p70β^{S6k}-binding partners.

Materials and Methods. HEK293 cells are transfected using lipofectAMINE (as recommended by manufacturer, Gibco-BRL) with pcDNA3 expression vectors encoding activated variants of p70β^{S6k} (T401D) and p70α^{S6k} (T412D). Binding partners that preferentially associate with p70β^{S6k} may be identified by comparing the profile of proteins precipitated from activated p70β^{S6k} expressing cells to the profile of activated p70α^{S6k} expressing cells and/or negative control cells. Cells transfected with the pcDNA3 plasmid alone, may be used as a negative control in this experiment. Two days after transfection, cells are lysed in extraction buffer: 50 mM Tris/HCl (pH8.0); 120 mM NaCl; 20 mM NaF; 20 mM b-glycerophosphate; 1 mM EDTA, (pH 8.0); 6

mM EGTA; 1% NP-40; 1 mM DTT. The following protease and phosphatase inhibitors are added to the extraction buffer just before cell lysis: 5 mM Benzamidine; 1 mM PMSF; 1mg/ml of aprotinin; 0.125 mM NaVO4; Pepstatin; and Leupeptin.

The resulting cell lysate is centrifuged at 14,000 rpm for 20 min at 4°C to remove the insoluble fraction. If the lysate is not used immediately it is stored at -80°C until needed. The protein concentration of the samples is measured using a Coomassie Protein Assay reagent (Pierce) at 595 nm. An equal amount of supernatant from each sample is added to fresh 1.5 ml tubes and the volumes is equalized using lysis buffer. Affinity purified anti-Glu antibody is added to the supernatant and incubated on the wheel for 1 hr at 4°C. Protein-G sepharose beads, pre-washed in lysis buffer, are used to bring down immune complexes.

After extensive washing in lysis buffer (4x), 2x sample buffer is added to the beads. Bound proteins are eluted from the beads by boiling and separated by SDS-PAGE electrophoresis. Separated proteins are silver stained and the pattern of associated proteins is analyzed.

Results. The pattern of associated proteins are compared between activated variants of $p70\beta^{S6k}$ and $p70\alpha^{S6k}$ kinases. Mutated variants of both kinases are transiently expressed in HEK293 cells as Glu-tag fusion proteins. The presence of a Glu-tag epitope at the N-terminus of $p70\beta^{S6k}$ and $p70\alpha^{S6k}$ allows specific immunoprecipitation of activated kinases from transfected cells. The skilled practioner will recognize that the Glu-tag fusion is not necessary to the invention and that similar results could be obtained with antibodies specific to each or both of the activated variants in the absence of a fusion epitope.

The skilled practioner will recognize that binding partners or polypeptides that preferentially bind to activated $P70\beta^{S6K}$ can be isolated by one or more standard techniques such as immunoprecipitation, hplc, fplc, column chromatography or preparative electrophoresis.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without

departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

WHAT IS CLAIMED:

1. An isolated nucleic acid molecule selected from the group consisting of a nucleic acid molecule comprising the sequence of SEQ ID No.1 (the nucleotide sequence of the $p70\beta^{S6k}$); and a nucleic acid molecule which encodes a $p70\beta^{S6k}$ and which hybridizes to a nucleic acid molecule having the sequence of SEQ ID No.1 under stringent conditions.

2. An isolated nucleic acid molecule which encodes a protein having SEQ ID No.2 (the $p70\beta^{S6k}$) or a protein having one or more conservative amino acid substitutions in SEQ ID No.2.

3. An isolated nucleic acid molecule selected from the group consisting of a nucleic acid molecule comprising the sequence of SEQ ID No.1 (the nucleotide sequence of the $p70\beta^{S6k}$); a nucleic acid molecule which encodes a $p70\beta^{S6k}$ and which hybridizes to a nucleic acid molecule having the sequence of SEQ ID No.1 under stringent conditions, and a nucleic acid molecule at least about 65% sequence identity with SEQ ID No.1.

4. An isolated nucleic acid molecule which encodes a protein having SEQ ID No.2 (the $p70\beta^{S6k}$), a protein with at least 75% identity to SEQ ID No.2, or a polypeptide fragment of SEQ ID No.2.

5. An isolated polypeptide which phosphorylates a ribosomal S6 protein and is encoded by a nucleic acid molecule of claim 1 or claim 2.

6. A method of identifying an agent which modulates $p70\beta^{S6k}$ mediated phosphorylation of a ribosomal S6 subunit comprising the steps of:

exposing $p70\beta^{S6k}$ and a ribosomal S6 subunit to the agent; and

determining whether the agent modulates $p70\beta^{S6k}$ mediated phosphorylation of the ribosomal S6 subunit.

7. A method of modulating protein synthesis or cellular proliferation comprising the step of administering an agent which modulates $p70\beta^{S6k}$ phosphorylation of a ribosomal S6 subunit.

8. A method of identifying an agent that modulates a kinase or a phosphatase induced regulation of $p70\beta^{S6k}$ activity comprising the steps of:

exposing $p70\beta^{S6k}$ and the kinase which phosphorylates $p70\beta^{S6k}$ to an agent; and

determining whether the agent modulates the kinase or the phosphatase induced regulation of $p70\beta^{S6k}$ activity.

9. The method of claim 8, wherein the kinase which phosphorylates $p70\beta^{S6k}$ is PKC.

10. A method of modulating protein synthesis or cellular proliferation comprising the step of administering an agent which modulates the phosphorylation of $p70\beta^{S6k}$.

11. A method of modulating cell cycle comprising the step of administering an agent which regulates the ability $p70\beta^{S6k}$ to bind with a ligand.

12. An antibody or antibody fragment which specifically binds to an epitope of $p70\beta^{S6k}$.

13. The antibody of claim 11, wherein the antibody is selected from the group consisting of a monoclonal antibody, human antibody, chimeric antibody, and humanized antibody.

14. An antibody of claim 11 wherein the epitope is a proline rich epitope of a $p70\beta^{S6k}$ protein.

15. A fusion protein comprising SEQ ID No.2 or a polypeptide fragment thereof fused to a heterologous protein.

16. A cell transformed with a nucleic acid molecule of any of claims 1-3.

17. A method of identifying a substrate of $p70\beta^{S6k}$ comprising the steps of:
exposing $p70\beta^{S6k}$ or a polypeptide fragment thereof to an agent; and
determining whether $p70\beta^{S6k}$ binds to the agent.

18. A method of identifying a substrate of $p70\beta^{S6k}$ comprising the steps of:
forming a mixture comprising $p70\beta^{S6k}$ and a candidate agent;
incubating said mixture under conditions conducive to phosphorylation by $p70\beta^{S6k}$; and
determining whether the candidate agent is phosphorylated.

19. A method of indentifying binding partners of $p70\beta^{S6K}$ comprising the step of incubating a first cellular extract with $p70\beta^{S6K}$, activated variants of $p70\beta^{S6K}$ or a fusion protein of claim 15.

20. The method of claim 19 further comprising incubating a second cellular extract with $p70\alpha^{S6K}$, activated variants of $p70\alpha^{S6K}$ or a fusion protein of $p70\alpha^{S6K}$ and comparing the first and second cellular extracts.

21. A method of indentifying binding partners of $p70\beta^{S6K}$ comprising the step of isolating a first a first cellular extract from a cell containing $p70\beta^{S6K}$, activated variants of $p70\beta^{S6K}$ or a fusion protein of claim 15.

22. The method of claim 19 further comprising isolating a second cellular extract from a cell containing $p70\alpha^{S6K}$, activated variants of $p70\alpha^{S6K}$ or a fusion protein of $p70\alpha^{S6K}$ and comparing the first and second cellular extracts.

23. An isolated polypeptide comprising an activated $p70\beta^{S6k}$.

24. The isolated polypeptide of claim 23 further comprising a mutation of Threonine 401 to Aspartic acid.

25. An isolated polypeptide that preferentially binds to an activated $p70\beta^{S6K}$ of claim 23.

26. The isolated polypeptide of claim 25 that preferentially binds to an activated $p70\beta^{S6k}$ of claim 24.

27. An antibody or antibody fragment that specifically binds to the isolated polypeptide of claims 25 or 26.

28. A method of determining whether a cell expresses aberrant cellular levels of $p70\beta^{S6k}$ comprising:
(a) determining the level of $p70\beta^{S6k}$ in a normal cell type;
(b) determining the level of $p70\beta^{S6k}$ in a test cell;
(c) comparing the level of $p70\beta^{S6k}$ in the normal cell to the $p70\beta^{S6k}$ level in the test cell.

29. The method of claim 28 wherein the level of $p70\beta^{S6k}$ is determined by finding the level $p70\beta^{S6k}$ RNA in a cell.

30. The method of claim 28, wherein the level of $p70\beta^{S6k}$ is determined by finding the level of $p70\beta^{S6k}$ protein in a cell.

31. A method of determining whether a cell expresses aberrant cellular levels of a $p70\beta^{S6k}$ binding partner comprising:
(a) determining the level of said binding partner in a normal cell;
(b) determining the level of said binding partner in a test cell;
(c) comparing the level of said binding partner in the normal cell to the binding partner level in the test cell.

32. A vector comprising the isolated nucleic acid of claim 2, operably linked to a promotor or transcription.

33. The vector of claim 32, further comprising one or more enhancers or upstream activating sequences.

34. The vector of claim 32, wherein the vector comprises pcDNA3.

35. A vector which encodes an activated $p70\beta^{S6k}$ of claims 23 or 24.

36. A DNA vector comprising a nucleic acid encoding a $p70\beta^{S6k}$ or an activated $p70\beta^{S6k}$ fusion protein.

```
kestrel% more p70aHum.pair
GAP of: p70aHum  check: 8297  from: 1  to: 2346

to: p70b,c153,DNA,full,final  check: 8147  from: 1  to: 1816

Symbol comparison table: /gcg/gcgsoft/gcgcore/data/rundata/nwsgapdna.cmp
CompCheck: 6876

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    Length Weight:  0.300   Average Mismatch:  0.000

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             Ratio:  0.563               Gaps:     7
Percent Similarity: 59.541   Percent Identity: 59.541
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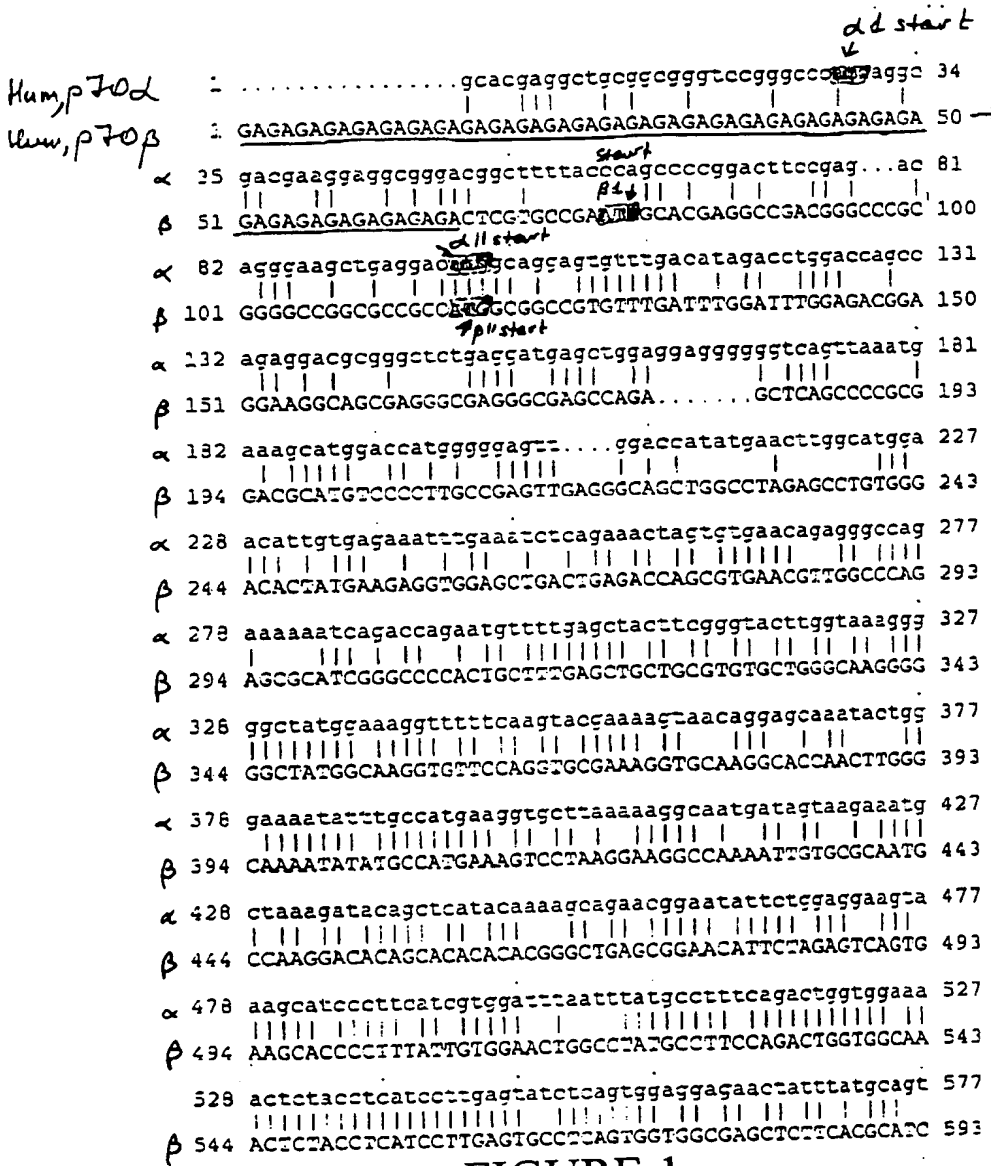

FIGURE 1

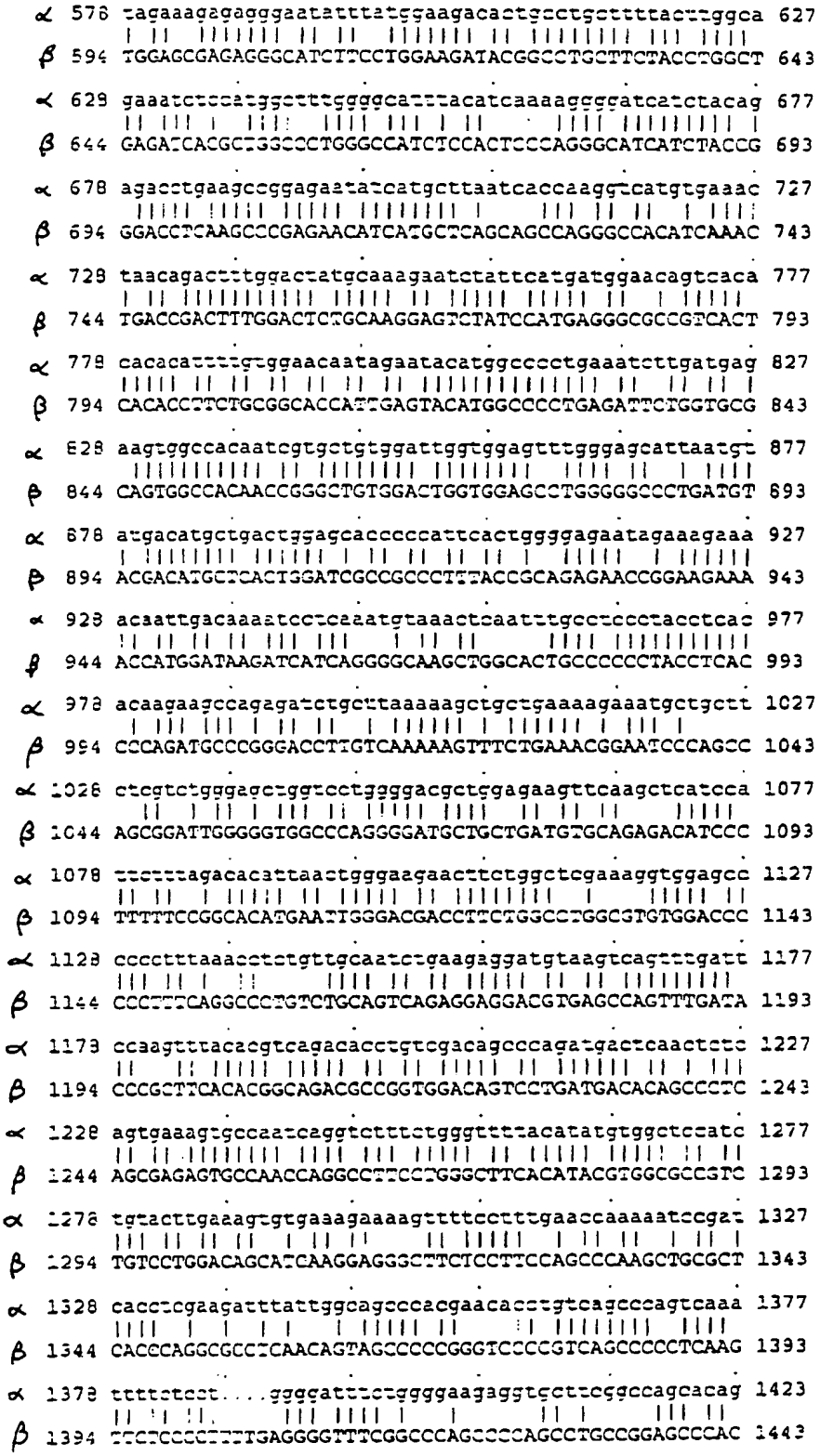

FIGURE 1 (cont'd)

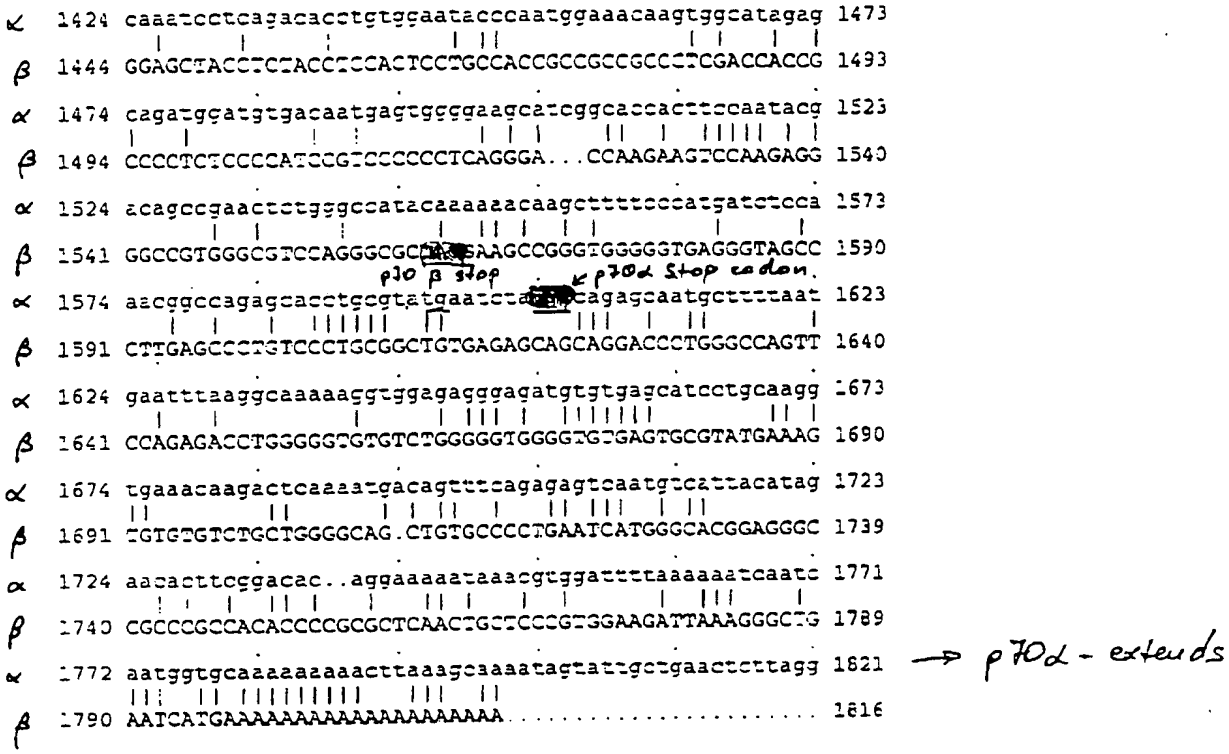

FIGURE 1 (cont'd)

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P70a      1  MRRRRRRDGFYPAPDFRDREAEDMAGVFDIDLD  33
p70b      1  ----------MARGRRARGAGAAMAAVFDLDLE  23

P70a     34  QPEDAGSEDELEEGGQLNESMDHGGVGPYELGM  66
p70b     24  TEEGSEGEGEPELSPADACPLAELRAAGLE-PV  55

P70a     67  EHCEKFEISETSVNRGPEKIRPECFELLRVLGK  99
p70b     56  GHYEEVELTETSVNVGPERIGPHCFELLRVLGK  88

P70a    100  GGYGKVFQVRKVTGANTGKIYAMKVLKKAMIVR 132
p70b     89  GGYGKVFQVRKVQGTNLGKIYAMKVLRKAKIVR 121

P70a    133  NAKDTAHTKAERNILEEVKHPFIVDLIYAFQTG 165
p70b    122  NAKDTAHTRAERNILESVKHPFIVELAYAFQTG 154

P70a    156  GKLYLILEYLSGGELFMQLEREGIFMEDTACFY 188
p70b    155  GKLYLILECLSGGELFTHLEREGIFLEDTACFY 187

P70a    199  LAEISMALGHLHQKGIIYRDLKPENIMLNHQGH 231
p70b    188  LAEITLALGHLHSQGIIYRDLKPENIMLSSQGH 220

P70a    232  VKLTDFGLCKESIHDGTVTHTFCGTIEYMAPEI 264
p70b    221  IKLTDFGLCKESIHEGAVTHTFCGTIEYMAPEI 253

P70a    265  LMRSGHNRAVDWWSLGALMYDMLTGASPFTGEN 297
p70b    254  LVRSGHNRAVDWWSLGALMYDMLTGSPPFTAEN 286

P70a    298  RKKTIDKILKCKLNLPPYLTQEARDLLKKLLKR 330
p70b    287  RKKTMDKIIRGKLALPPYLTPDARDLVKKFLKR 319

P70a    331  NAASRLGAGPGDAGEVQAHPFFRHINWEELARR 363
p70b    320  NPSQRIGGGPGDAADVQRHPFFRHMNWDDLLAW 352

P70a    364  KVEPPFKPLLQSEEDVSQFDSKFTRQTPVDSPD 396
p70b    353  RVDPPFRPCLQSEEDVSQFDTRFTRQTPVDSPD 385

P70a    397  DSTLSESANGVFLGFTYVAPSVLESVKEKFSFE 429
p70b    386  DTALSESANGAFLGFTYVAPSVLDSIKEGFSFQ 418

P70a    430  PKIRSPRRFIGSPRTPVSPVKFSP-GDFW-GRG 460
p70b    419  PKLRSPRRLNSSPRVPVSPLKFSPFEGFRPSPS 451

P70a    461  ASASTANPQTPVEYPMETSGIEQMDVTMSGEAS 493
p70b    452  LPEPTELPLPPLLPPPPPSTTAPLPIRPPSGTK 484

P70a    494  APLPIRQPNSGPYKKQAFPMISKRPEHLRMNL 525
p70b    485  KSKRGRGRPGR                       495
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FIGURE 2A

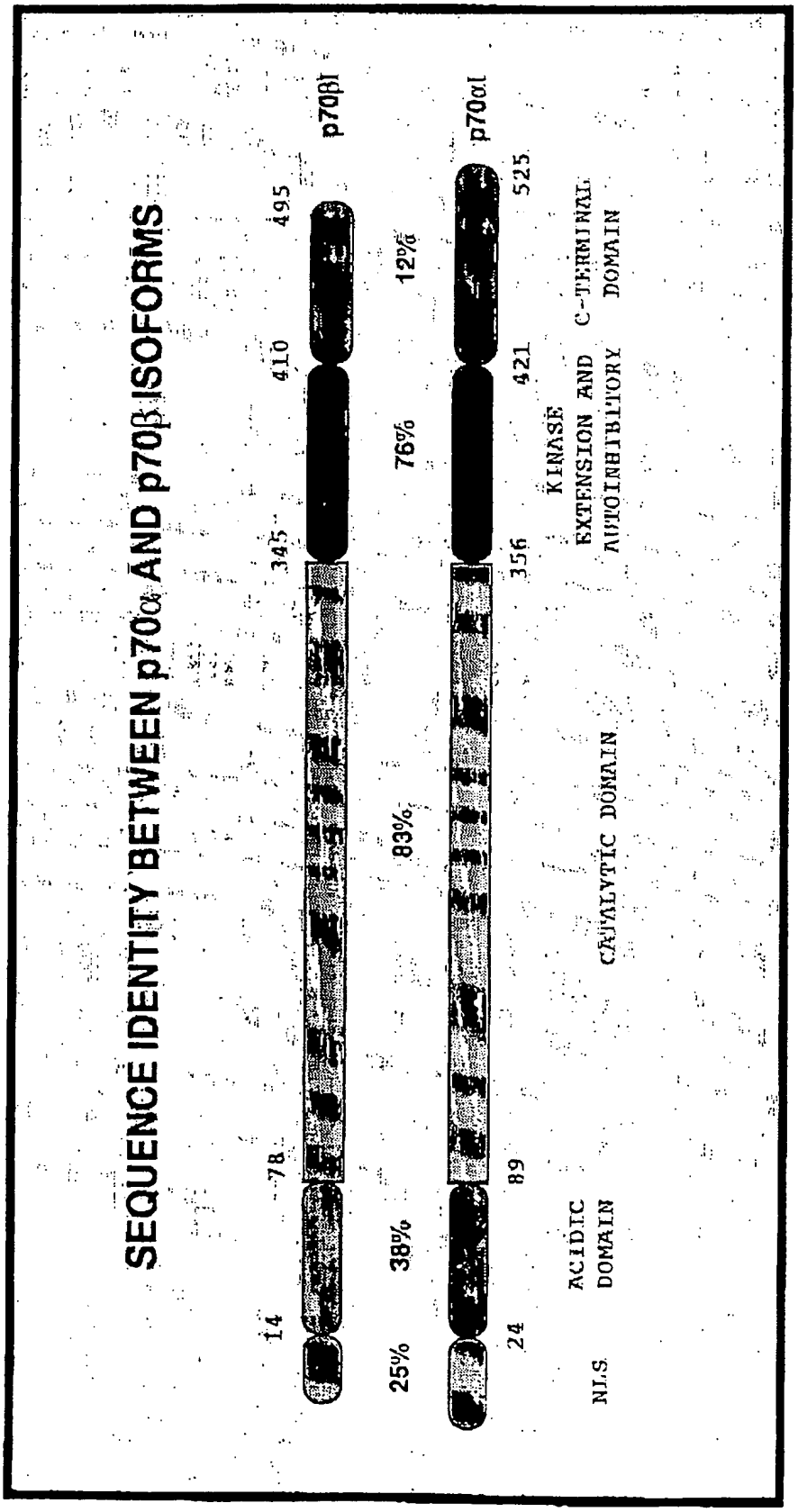

SEQUENCE IDENTITY BETWEEN p70α AND p70β ISOFORMS
p70βI
p70αI
14
78
345
410
495
24
89
356
421
525
25%
38%
83%
76%
12%
NLS
ACIDIC DOMAIN
CATALYTIC DOMAIN
KINASE EXTENSION AND AUTOINHIBITORY
C-TERMINAL DOMAIN

FIGURE 2B

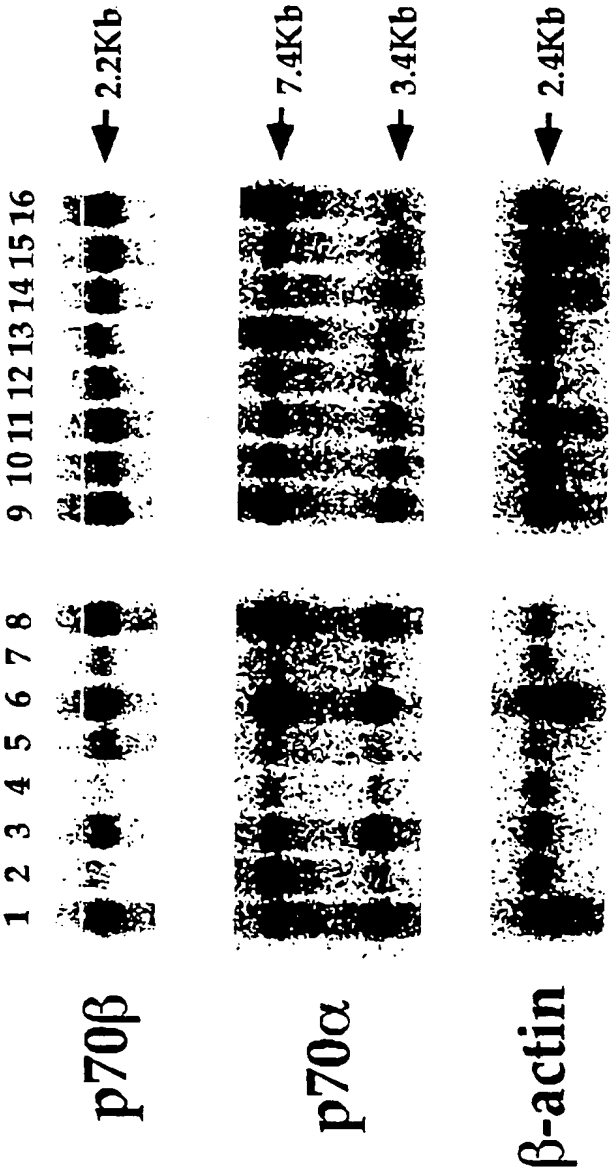

1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
2.2Kb
7.4Kb
3.4Kb
2.4Kb
p70β
p70α
β-actin

FIGURE 3A

Expression pattern of the p70β mRNAs in tumour cell lines

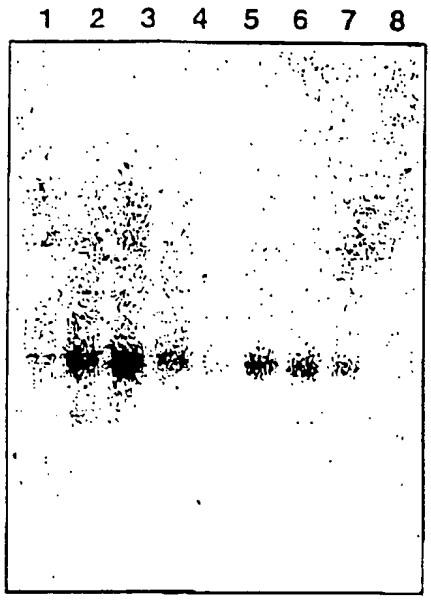


FIGURE 3B

1 Promyelocytic leukemia HL-60
2 HeLa cell S3
3 chronic myelogenous leukemia K562
4 Lymphoblastic leukemia MOLT-4
5 Burkitt's lymphoma Raji
6 colorectal adenocarcinoma SW480
7 Lung carcinoma A549
8 Melanoma G361

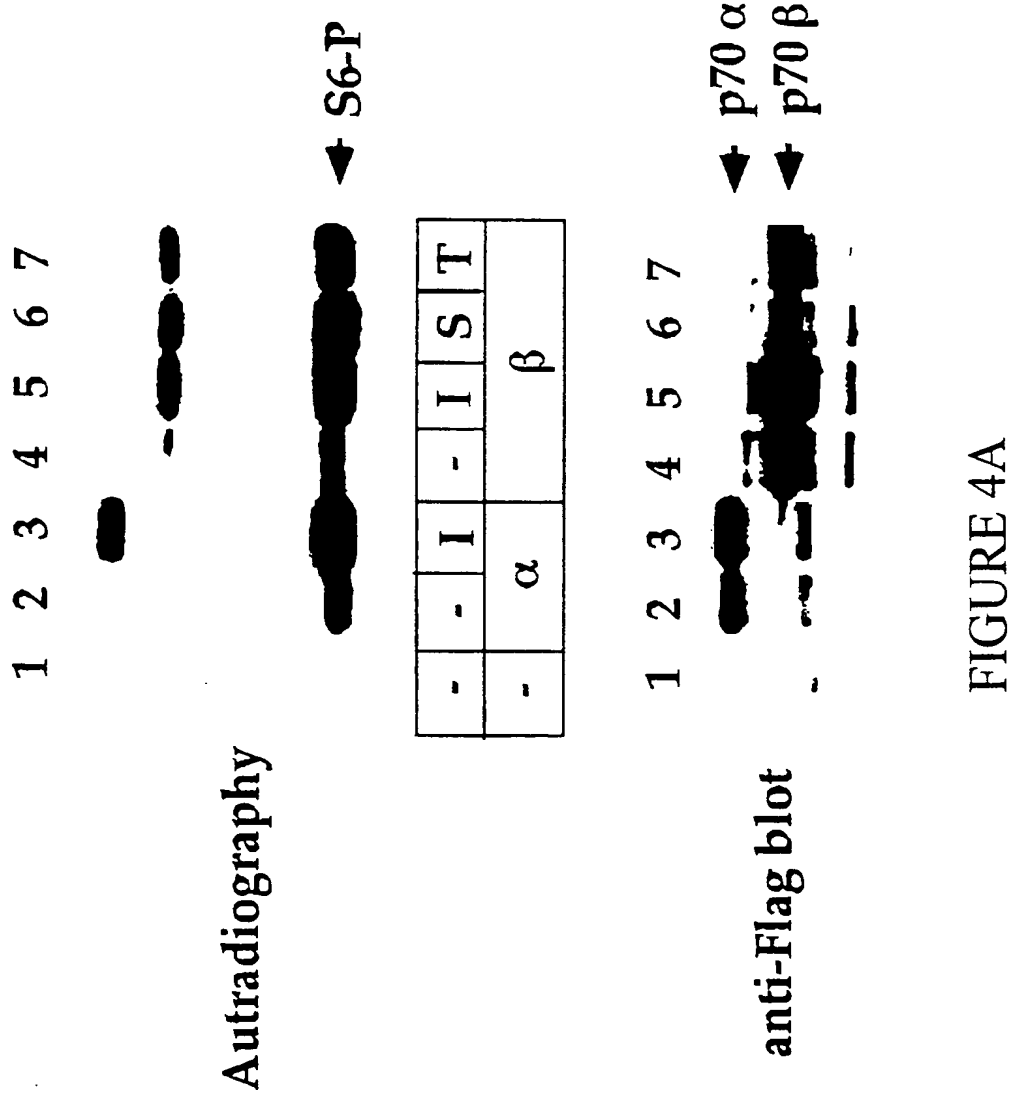

1 2 3 4 5 6 7
Autradiography
S6-P
- - I - I S T
- α β
1 2 3 4 5 6 7
anti-Flag blot
p70 α
p70 β

FIGURE 4A

PHOSPHORYLATION OF THE RIBOSOMAL S6 PROTEIN
C-TERMINAL PEPTIDES BY p70α AND β KINASES

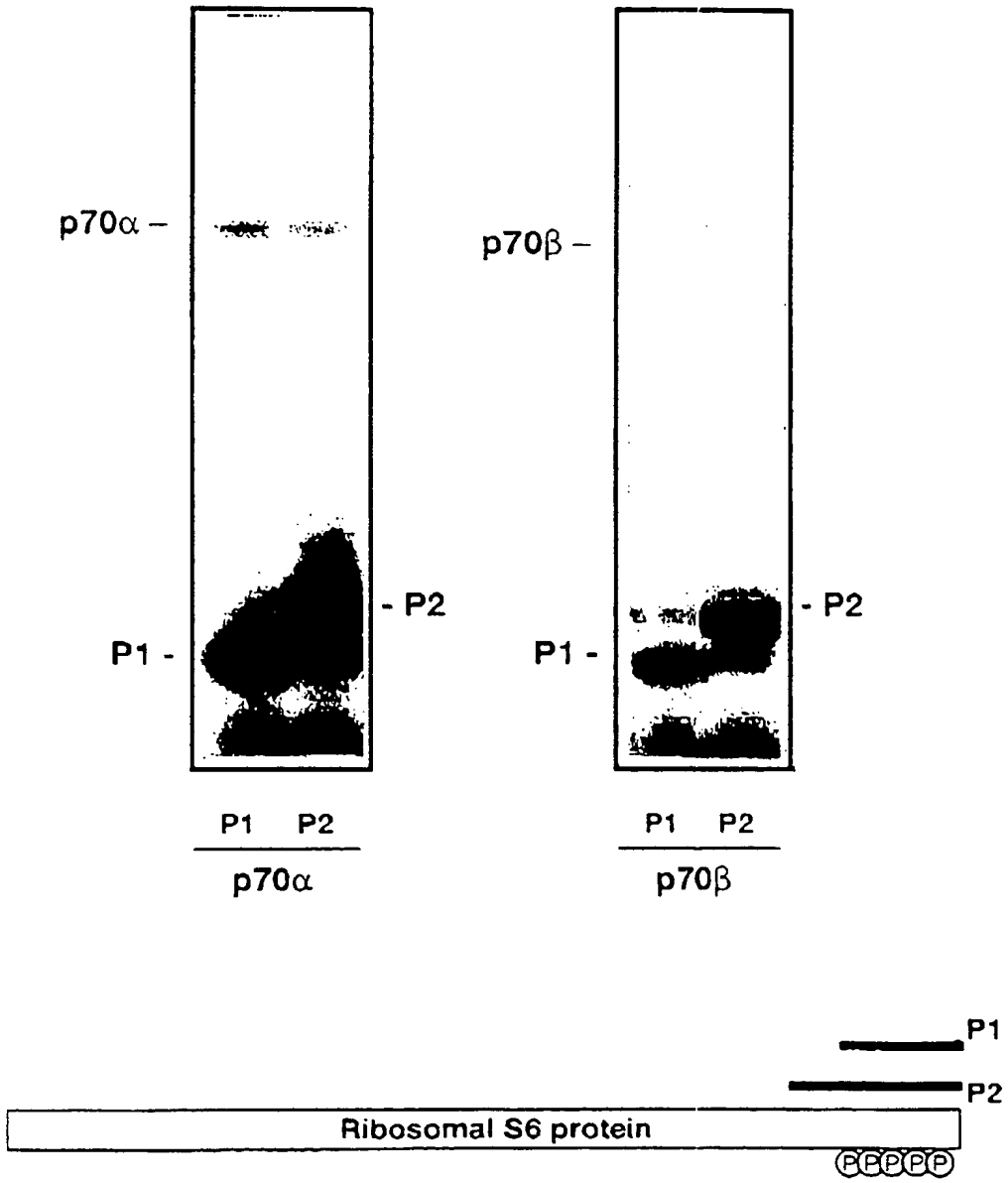
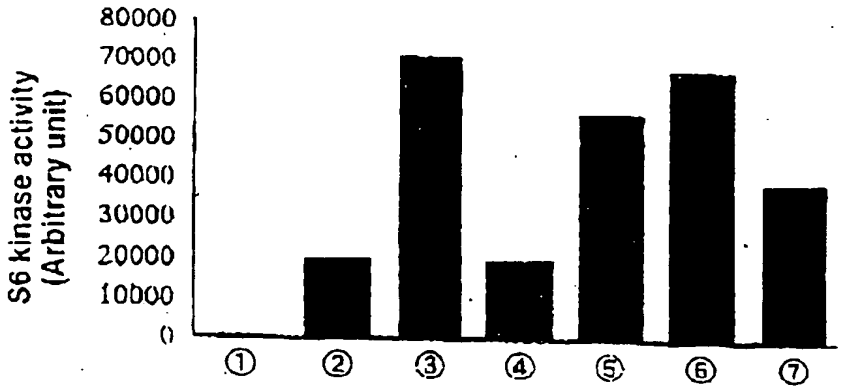


FIGURE 4B

ACTIVATION THE p70α and β KINASES
IN RESPONSE TO VARIOUS STIMULI *in vivo*



1 mock transfection

2 p70α (starved and non treated)

3 p70α (starved and insulin stimulated)

4 p70β (starved and non treated)

5 p70β (starved and insulin stimulated)

6 p70β (starved and serum stimulated)

7 p70β (starved and TPA stimulated)

FIGURE 5A

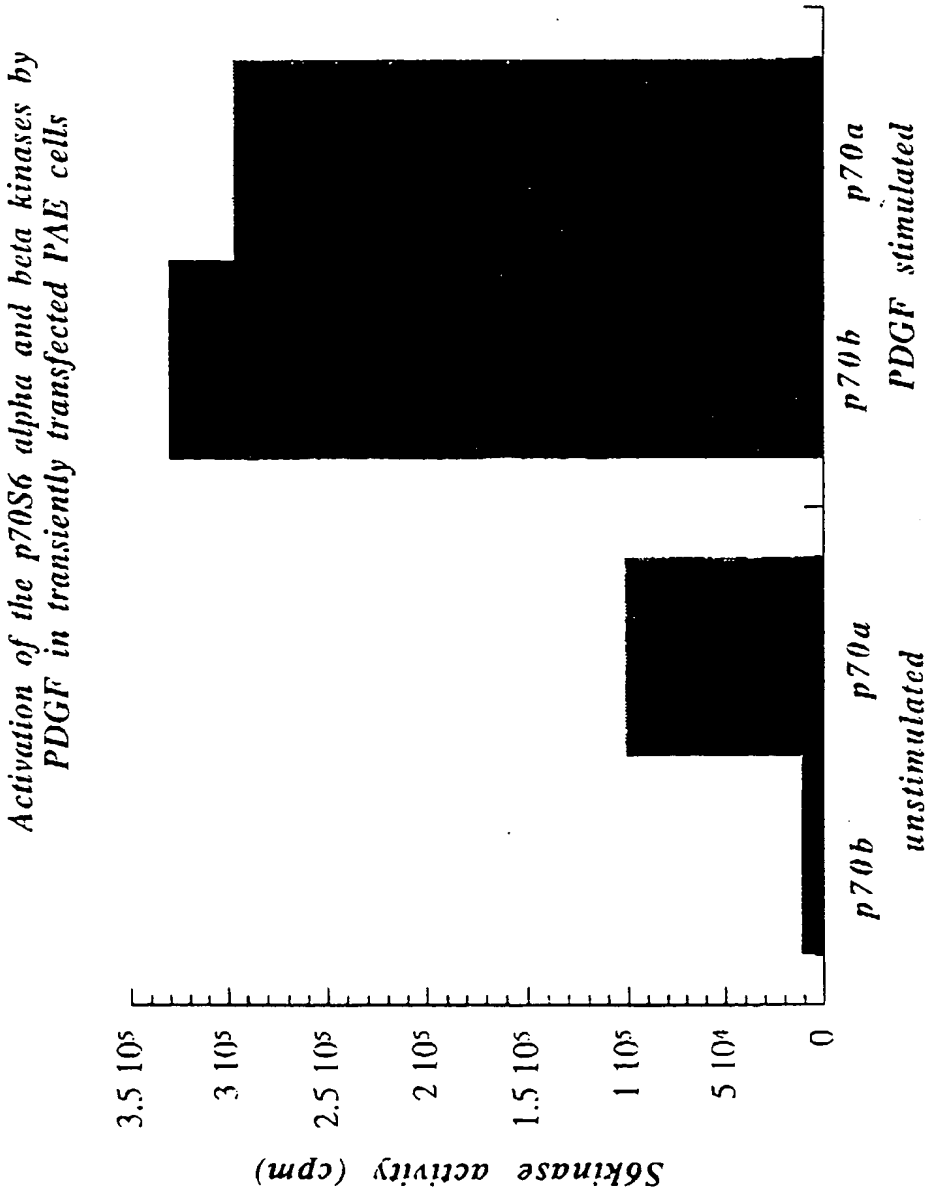

Activation of the p70S6 alpha and beta kinases by PDGF in transiently transfected PAE cells
S6kinase activity (cpm)
3.5 10^5
3 10^5
2.5 10^5
2 10^5
1.5 10^5
1 10^5
5 10^4
0
p70b
p70a
unstimulated
p70b
p70a
PDGF stimulated

FIGURE 5B

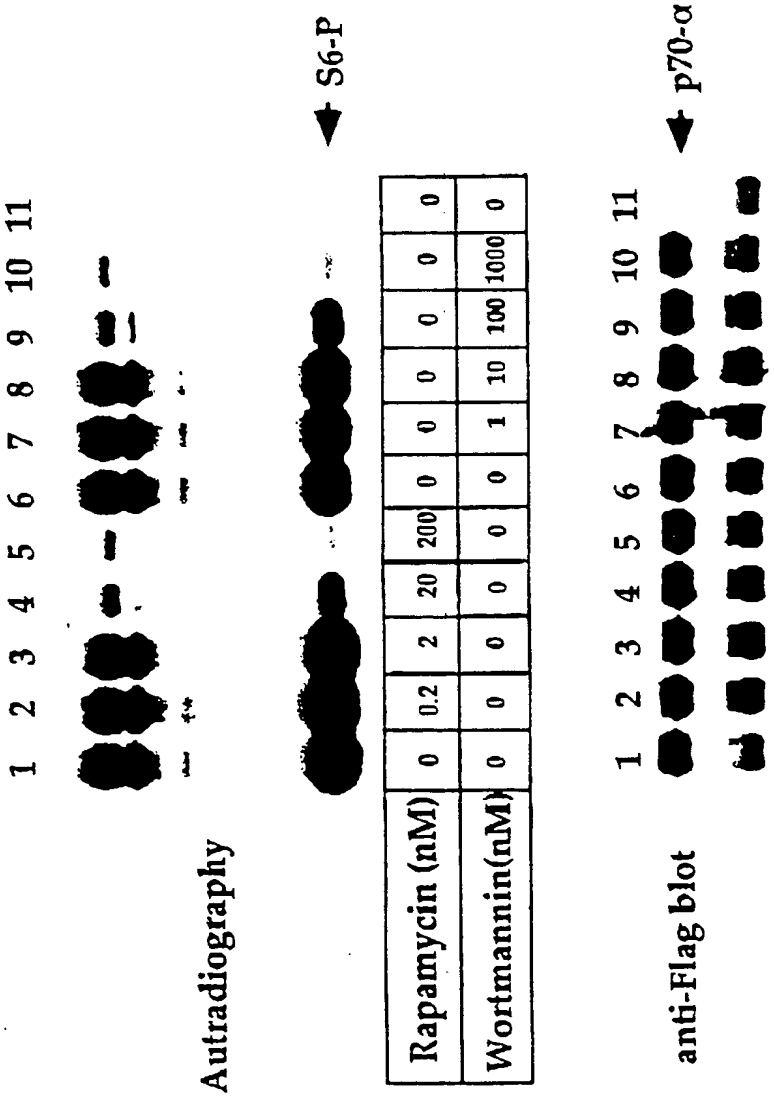

1 2 3 4 5 6 7 8 9 10 11
Autradiography
S6-P
Rapamycin (nM) 0 0 0.2 2 20 200 0 0 0 0 0
Wortmannin(nM) 0 0 0 0 0 0 1 10 100 1000 0
1 2 3 4 5 6 7 8 9 10 11
anti-Flag blot
p70-α

FIGURE 6A

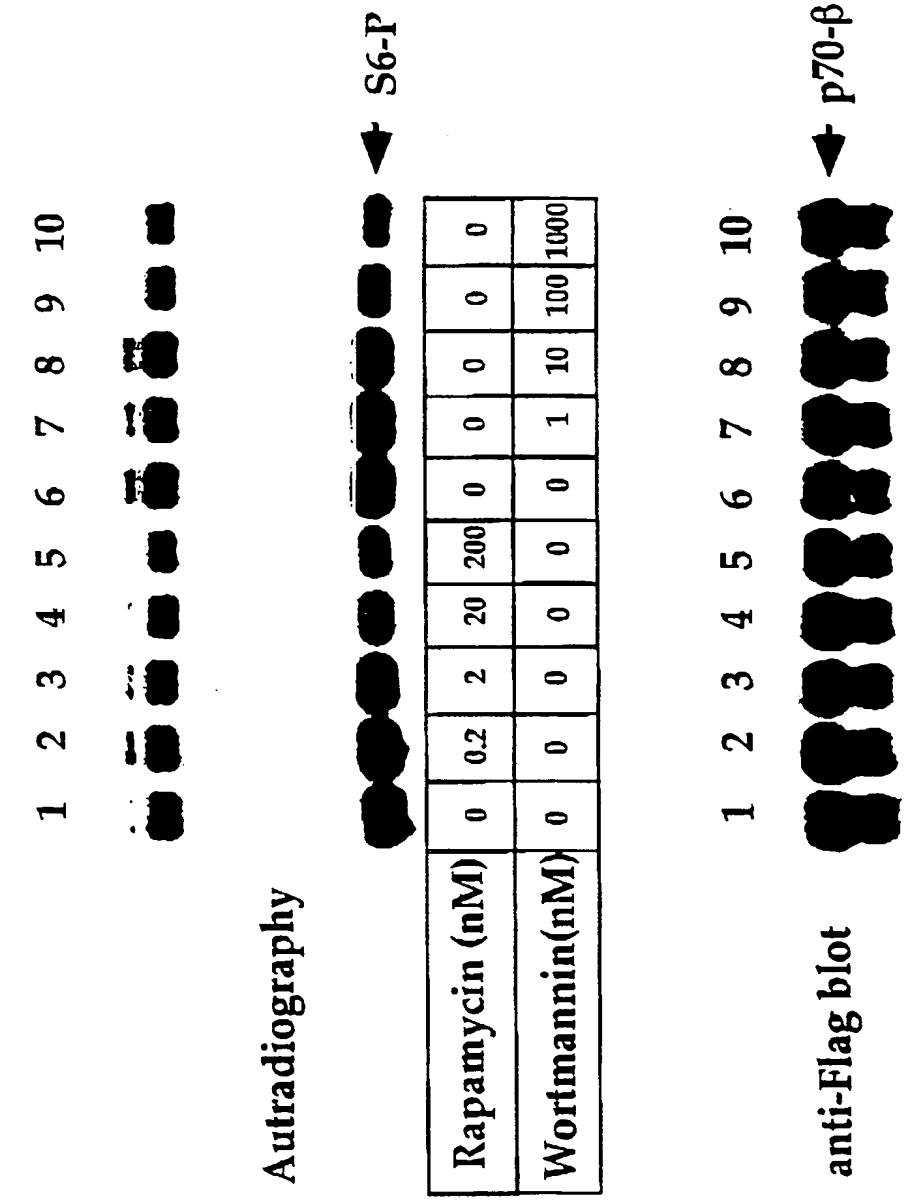

1 2 3 4 5 6 7 8 9 10
Autradiography
S6-P
Rapamycin (nM) 0 0.2 2 20 200 0 0 0 0 0
Wortmannin(nM) 0 0 0 0 0 0 1 10 100 1000
1 2 3 4 5 6 7 8 9 10
anti-Flag blot
p70-β

FIGURE 6B

INTERACTION OF P70S6K ß WITH DIFFERENT GST/SH3 FUSION PROTEINS IN VITRO

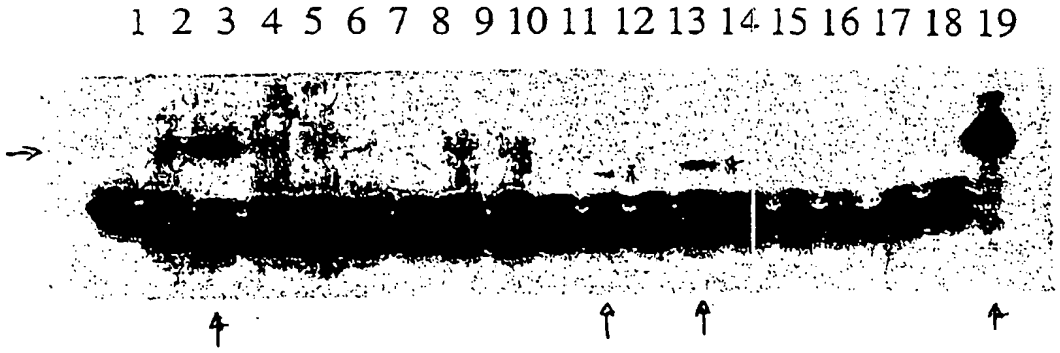
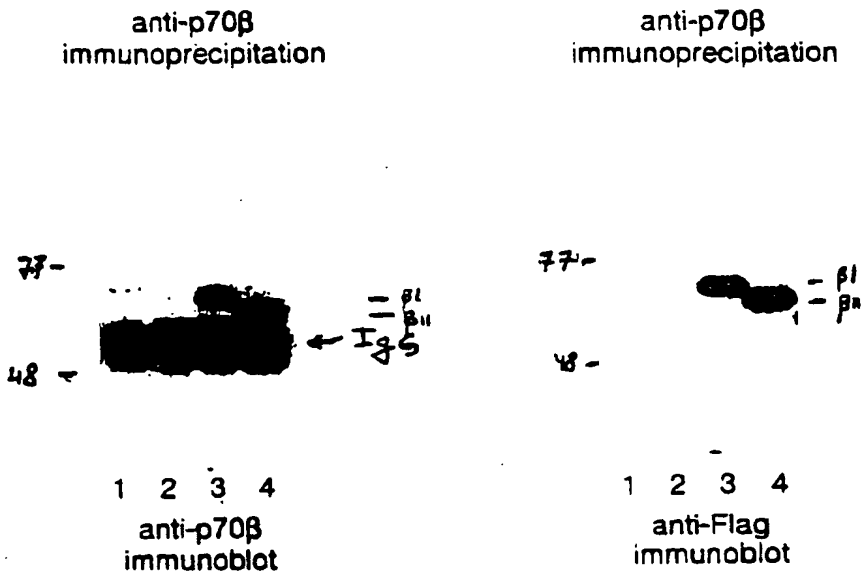


FIGURE 7

Immunoprecipation and Western blot analysis of the p70βI and βII transiently overexpressed in Hek 293 cells with anti-p70 antibodies.



1. mock transfection
2. Flag-p70αI
3. Flag-p70βI
4. Flag-p70βII

FIGURE 8

Fig 12

Comparison between activity of P70α wt and P70α 412

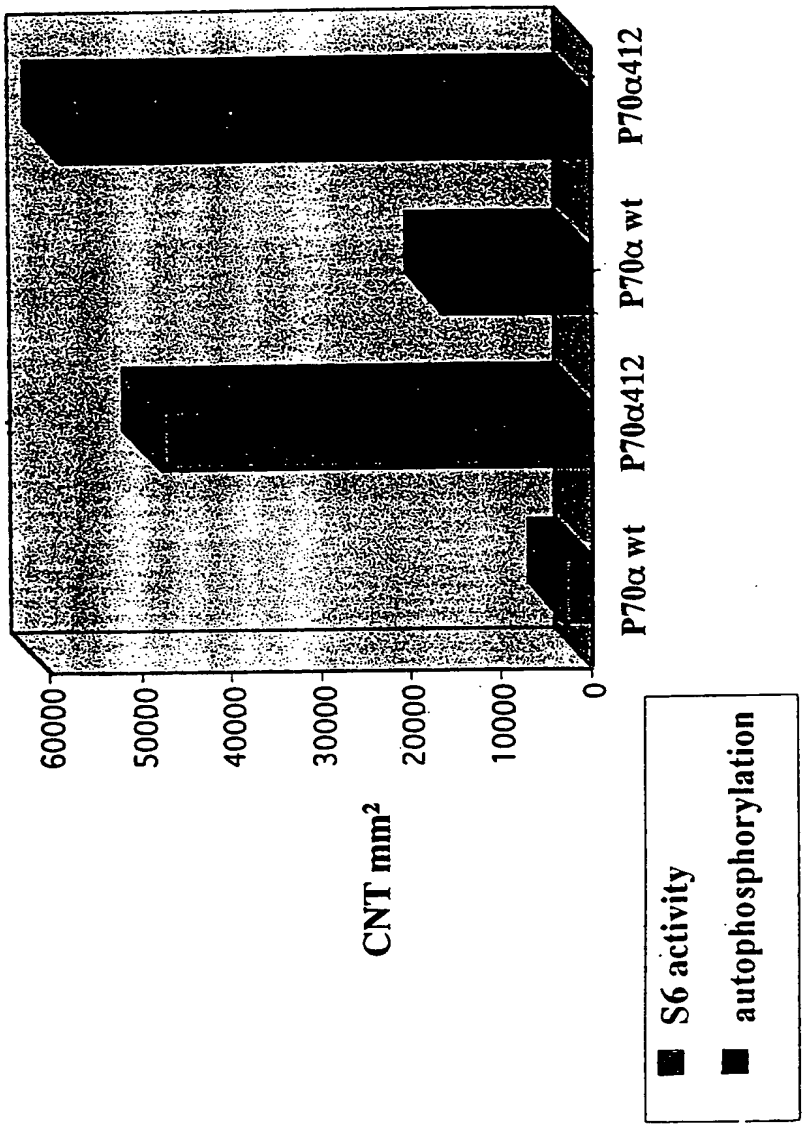


Fig. 11

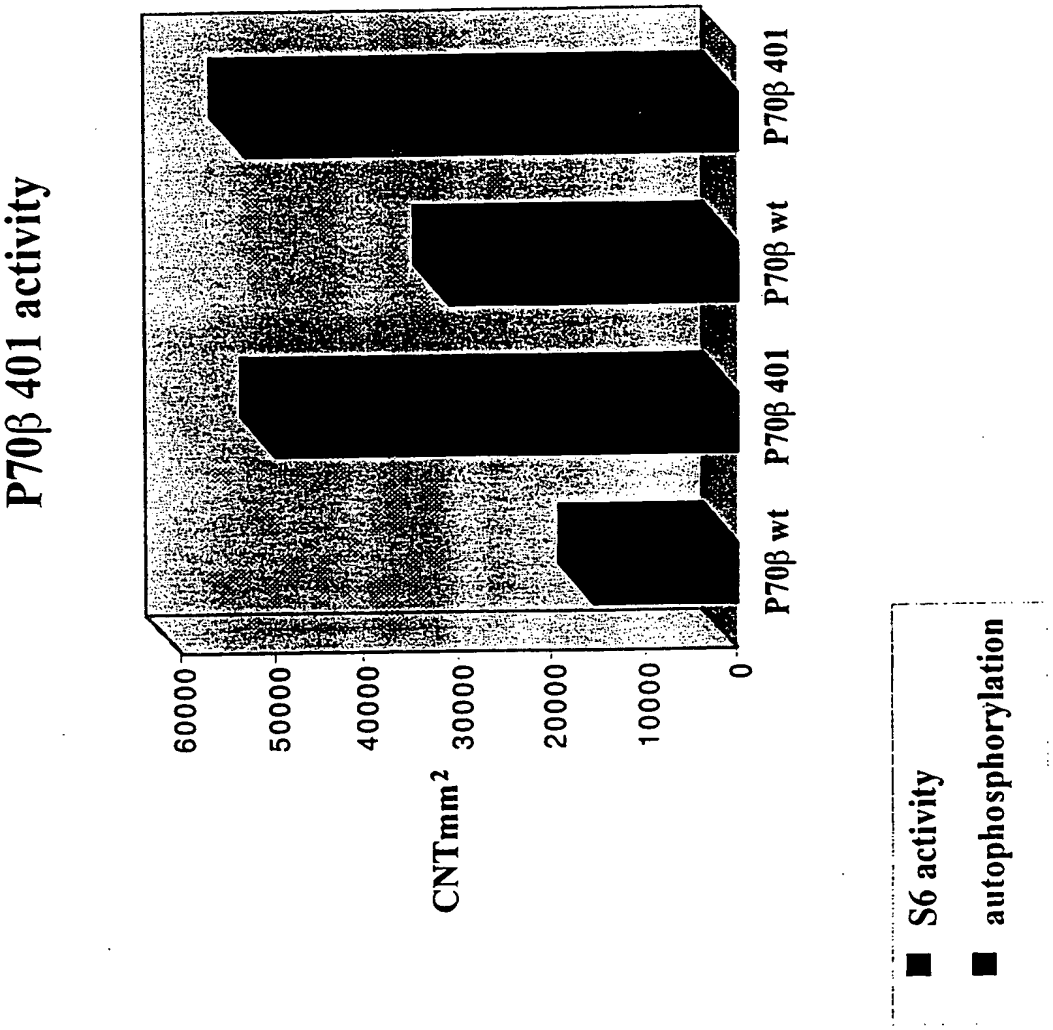

P70β 401 activity
60000
50000
40000
30000
20000
10000
0
CNTmm²
P70β wt
P70β 401
P70β wt
P70β 401
S6 activity
autophosphorylation

Fig 1C

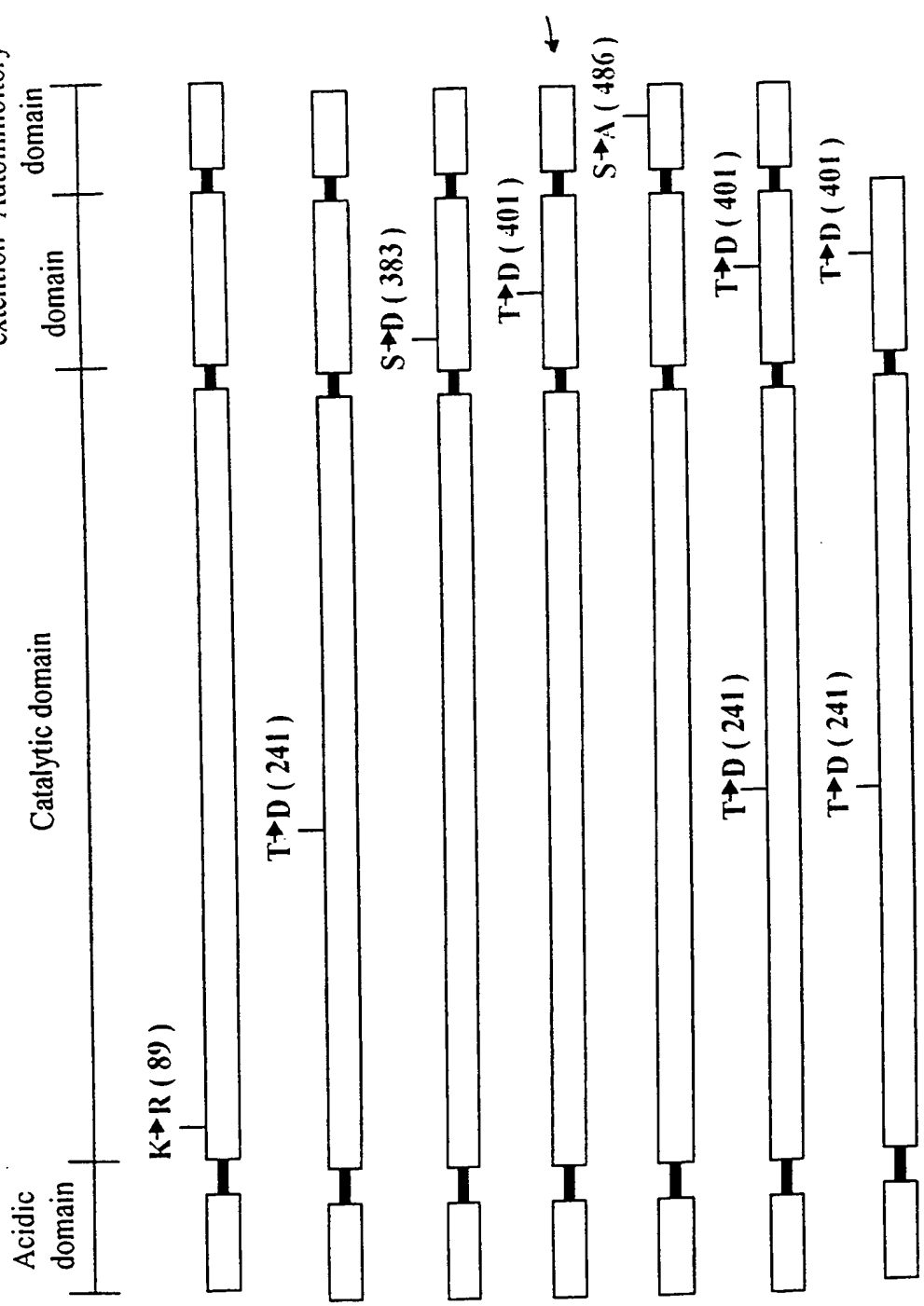
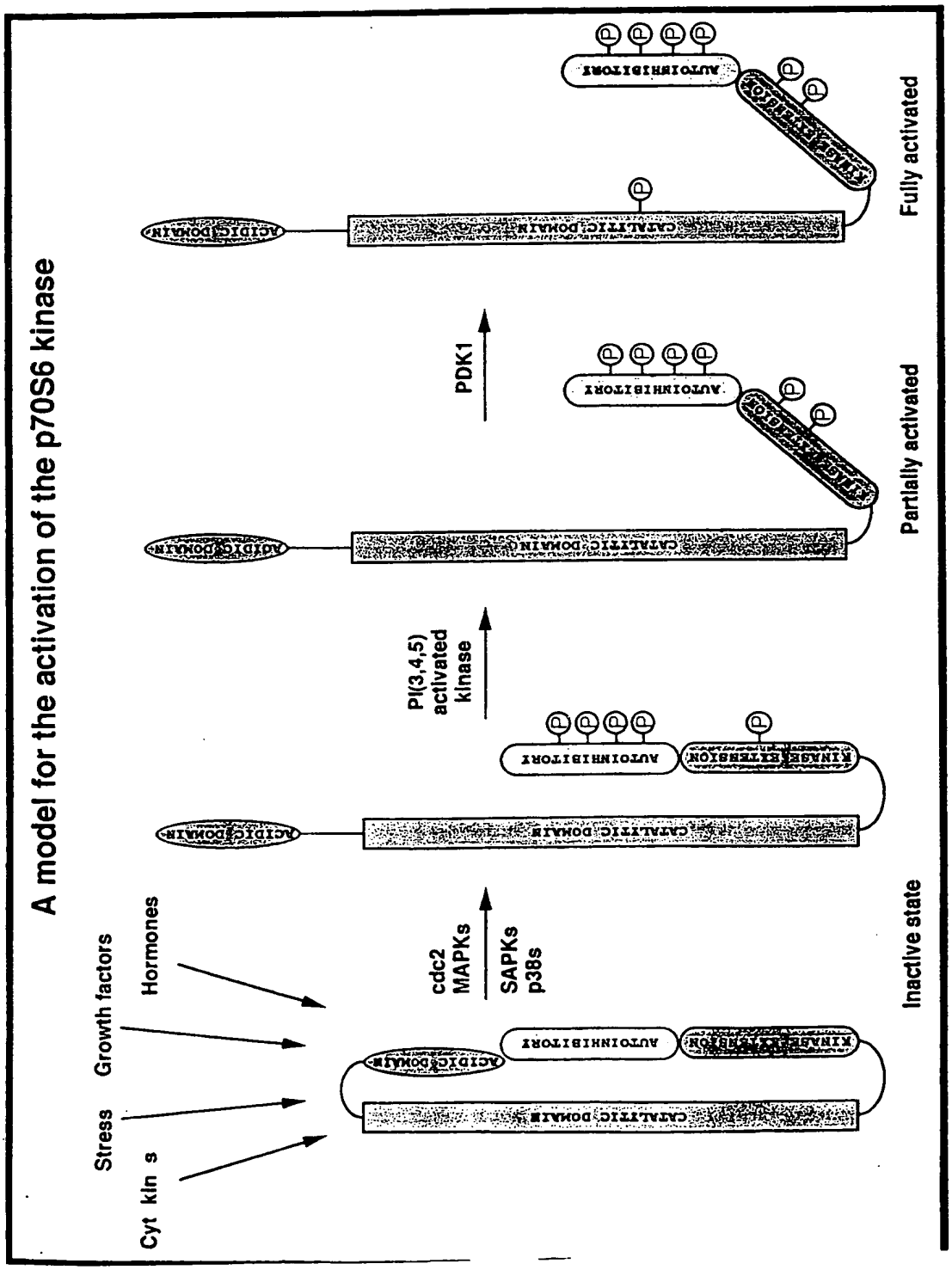

p70 β Mutations
Acidic domain
Catalytic domain
Kinase extention domain
Autoinhibitory domain
K→R (89)
T→D (241)
S→D (383)
T→D (401)
S→A (486)
T→D (241)
T→D (401)
T→D (241)
T→D (401)

Fig. 4


A model for the activation of the p70S6 kinase
Cyt kln s
Stress
Growth factors
Hormones
cdc2
MAPKs
SAPKs
p38s
PI(3,4,5)
activated
kinase
PDK1
Inactive state
Partially activated
Fully activated

SEQUENCE LISTING

<110> Ludwig Institute for Cancer Research

<110> Gout, Ivan
Hara, Kenta
Waterfield, Michael
Yonezawa, Kazu

<120> Identification and Functional Characterization of a Novel Ribosomal S6 Protein Kinase

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Ala Met Ala Ala Val Phe Asp Leu Asp Leu Glu Thr Glu Glu Gly Ser
15 20 25

gag ggc gag ggc gag cca gag ctc agc ccc gcg gac gca tgt ccc ctt 208
Glu Gly Glu Gly Glu Pro Glu Leu Ser Pro Ala Asp Ala Cys Pro Leu
30 35 40

gcc gag ttg agg gca gct ggc cta gag cct gtg gga cac tat gaa gag 256
Ala Glu Leu Arg Ala Ala Gly Leu Glu Pro Val Gly His Tyr Glu Glu
45 50 55 60

gtg gag ctg act gag acc agc gtg aac gtt ggc cca gag cgc atc ggg 304
Val Glu Leu Thr Glu Thr Ser Val Asn Val Gly Pro Glu Arg Ile Gly
65 70 75

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Pro His Cys Phe Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly
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Lys Val Phe Gln Val Arg Lys Val Gln Gly Thr Asn Leu Gly Lys Ile
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tat gcc atg aaa gtc cta agg aag gcc aaa att gtg cgc aat gcc aag   448
Tyr Ala Met Lys Val Leu Arg Lys Ala Lys Ile Val Arg Asn Ala Lys
    110                 115                 120

gac aca gca cac aca cgg gct gag cgg aac att cta gag tca gtg aag   496
Asp Thr Ala His Thr Arg Ala Glu Arg Asn Ile Leu Glu Ser Val Lys
125                 130                 135                 140

cac ccc ttt att gtg gaa ctg gcc tat gcc ttc cag act ggt ggc aaa   544
His Pro Phe Ile Val Glu Leu Ala Tyr Ala Phe Gln Thr Gly Gly Lys
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Leu Tyr Leu Ile Leu Glu Cys Leu Ser Gly Gly Glu Leu Phe Thr His
            160                 165                 170

ctg gag cga gag ggc atc ttc ctg gaa gat acg gcc tgc ttc tac ctg   640
Leu Glu Arg Glu Gly Ile Phe Leu Glu Asp Thr Ala Cys Phe Tyr Leu
        175                 180                 185

gct gag atc acg ctg gcc ctg ggc cat ctc cac tcc cag ggc atc atc   688
Ala Glu Ile Thr Leu Ala Leu Gly His Leu His Ser Gln Gly Ile Ile
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tac cgg gac ctc aag ccc gag aac atc atg ctc agc agc cag ggc cac   736
Tyr Arg Asp Leu Lys Pro Glu Asn Ile Met Leu Ser Ser Gln Gly His
205                 210                 215                 220

atc aaa ctg acc gac ttt gga ctc tgc aag gag tct atc cat gag ggc   784
Ile Lys Leu Thr Asp Phe Gly Leu Cys Lys Glu Ser Ile His Glu Gly
                225                 230                 235

gcc gtc act cac acc ttc tgc ggc acc att gag tac atg gcc cct gag   832
Ala Val Thr His Thr Phe Cys Gly Thr Ile Glu Tyr Met Ala Pro Glu
            240                 245                 250

att ctg gtg cgc agt ggc cac aac cgg gct gtg gac tgg tgg agc ctg   880
Ile Leu Val Arg Ser Gly His Asn Arg Ala Val Asp Trp Trp Ser Leu
        255                 260                 265

ggg gcc ctg atg tac gac atg ctc act gga tcg ccg ccc ttt acc gca   928
Gly Ala Leu Met Tyr Asp Met Leu Thr Gly Ser Pro Pro Phe Thr Ala
    270                 275                 280

gag aac cgg aag aaa acc atg gat aag atc atc agg ggc aag ctg gca   976
Glu Asn Arg Lys Lys Thr Met Asp Lys Ile Ile Arg Gly Lys Leu Ala
285                 290                 295                 300
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ctg aaa cgg aat ccc agc cag cgg att ggg ggt ggc cca ggg gat gct   1072
Leu Lys Arg Asn Pro Ser Gln Arg Ile Gly Gly Gly Pro Gly Asp Ala
            320                 325                 330

gct gat gtg cag aga cat ccc ttt ttc cgg cac atg aat tgg gac gac   1120
Ala Asp Val Gln Arg His Pro Phe Phe Arg His Met Asn Trp Asp Asp
        335                 340                 345

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Leu Leu Ala Trp Arg Val Asp Pro Pro Phe Arg Pro Cys Leu Gln Ser
    350                 355                 360

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Glu Glu Asp Val Ser Gln Phe Asp Thr Arg Phe Thr Arg Gln Thr Pro
365                 370                 375                 380

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Val Asp Ser Pro Asp Asp Thr Ala Leu Ser Glu Ser Ala Asn Gln Ala
                385                 390                 395

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Phe Leu Gly Phe Thr Tyr Val Ala Pro Ser Val Leu Asp Ser Ile Lys
            400                 405                 410

gag ggc ttc tcc ttc cag ccc aag ctg cgc tca ccc agg cgc ctc aac   1360
Glu Gly Phe Ser Phe Gln Pro Lys Leu Arg Ser Pro Arg Arg Leu Asn
        415                 420                 425

agt agc ccc cgg gtc ccc gtc agc ccc ctc aag ttc tcc cct ttt gag   1408
Ser Ser Pro Arg Val Pro Val Ser Pro Leu Lys Phe Ser Pro Phe Glu
    430                 435                 440

ggg ttt cgg ccc agc ccc agc ctg ccg gag ccc acg gag cta cct cta   1456
Gly Phe Arg Pro Ser Pro Ser Leu Pro Glu Pro Thr Glu Leu Pro Leu
445                 450                 455                 460

cct cca ctc ctg cca ccg ccg ccg ccc tcg acc acc gcc cct ctc ccc   1504
Pro Pro Leu Leu Pro Pro Pro Pro Pro Ser Thr Thr Ala Pro Leu Pro
                465                 470                 475

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Ile Arg Pro Pro Ser Gly Thr Lys Lys Ser Lys Arg Gly Arg Gly Arg
            480                 485                 490

cca ggg cgc taggaagccg ggtgggggtg agggtagccc ttgagccctg           1601
Pro Gly Arg
        495

tccctgcggc tgtgagagca gcaggaccct gggccagttc cagagacctg ggggtgtgtc 1661

tgggggtggg gtgtgagtgc gtatgaaagt gtgtgtctgc tggggcagct gtgcccctga 1721
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             20                  25                  30

Glu Pro Glu Leu Ser Pro Ala Asp Ala Cys Pro Leu Ala Glu Leu Arg
         35                  40                  45

Ala Ala Gly Leu Glu Pro Val Gly His Tyr Glu Glu Val Glu Leu Thr
     50                  55                  60

Glu Thr Ser Val Asn Val Gly Pro Glu Arg Ile Gly Pro His Cys Phe
 65                  70                  75                  80

Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly Lys Val Phe Gln
                 85                  90                  95

Val Arg Lys Val Gln Gly Thr Asn Leu Gly Lys Ile Tyr Ala Met Lys
            100                 105                 110

Val Leu Arg Lys Ala Lys Ile Val Arg Asn Ala Lys Asp Thr Ala His
        115                 120                 125

Thr Arg Ala Glu Arg Asn Ile Leu Glu Ser Val Lys His Pro Phe Ile
    130                 135                 140

Val Glu Leu Ala Tyr Ala Phe Gln Thr Gly Gly Lys Leu Tyr Leu Ile
145                 150                 155                 160

Leu Glu Cys Leu Ser Gly Gly Glu Leu Phe Thr His Leu Glu Arg Glu
                165                 170                 175

Gly Ile Phe Leu Glu Asp Thr Ala Cys Phe Tyr Leu Ala Glu Ile Thr
            180                 185                 190

Leu Ala Leu Gly His Leu His Ser Gln Gly Ile Ile Tyr Arg Asp Leu
        195                 200                 205

Lys Pro Glu Asn Ile Met Leu Ser Ser Gln Gly His Ile Lys Leu Thr
    210                 215                 220
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225                 230                 235                 240

Thr Phe Cys Gly Thr Ile Glu Tyr Met Ala Pro Glu Ile Leu Val Arg
                245                 250                 255

Ser Gly His Asn Arg Ala Val Asp Trp Trp Ser Leu Gly Ala Leu Met
            260                 265                 270

Tyr Asp Met Leu Thr Gly Ser Pro Pro Phe Thr Ala Glu Asn Arg Lys
        275                 280                 285

Lys Thr Met Asp Lys Ile Ile Arg Gly Lys Leu Ala Leu Pro Pro Tyr
    290                 295                 300

Leu Thr Pro Asp Ala Arg Asp Leu Val Lys Lys Phe Leu Lys Arg Asn
305                 310                 315                 320

Pro Ser Gln Arg Ile Gly Gly Gly Pro Gly Asp Ala Ala Asp Val Gln
                325                 330                 335

Arg His Pro Phe Phe Arg His Met Asn Trp Asp Asp Leu Leu Ala Trp
            340                 345                 350

Arg Val Asp Pro Pro Phe Arg Pro Cys Leu Gln Ser Glu Glu Asp Val
        355                 360                 365

Ser Gln Phe Asp Thr Arg Phe Thr Arg Gln Thr Pro Val Asp Ser Pro
    370                 375                 380

Asp Asp Thr Ala Leu Ser Glu Ser Ala Asn Gln Ala Phe Leu Gly Phe
385                 390                 395                 400

Thr Tyr Val Ala Pro Ser Val Leu Asp Ser Ile Lys Glu Gly Phe Ser
                405                 410                 415

Phe Gln Pro Lys Leu Arg Ser Pro Arg Arg Leu Asn Ser Ser Pro Arg
            420                 425                 430

Val Pro Val Ser Pro Leu Lys Phe Ser Pro Phe Glu Gly Phe Arg Pro
        435                 440                 445

Ser Pro Ser Leu Pro Glu Pro Thr Glu Leu Pro Leu Pro Pro Leu Leu
    450                 455                 460

Pro Pro Pro Pro Pro Ser Thr Thr Ala Pro Leu Pro Ile Arg Pro Pro
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Ser Gly Thr Lys Lys Ser Lys Arg Gly Arg Gly Arg Pro Gly Arg
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Gly Val Phe Asp Ile Asp Leu Asp Gln Pro Glu Asp Ala Gly Ser Glu
                 30                  35                  40

gat gag ctg gag gag ggg ggt cag tta aat gaa agc atg gac cat ggg   198
Asp Glu Leu Glu Glu Gly Gly Gln Leu Asn Glu Ser Met Asp His Gly
             45                  50                  55

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Gly Val Gly Pro Tyr Glu Leu Gly Met Glu His Cys Glu Lys Phe Glu
         60                  65                  70

atc tca gaa act agt gtg aac aga ggg cca gaa aaa atc aga cca gaa   294
Ile Ser Glu Thr Ser Val Asn Arg Gly Pro Glu Lys Ile Arg Pro Glu
     75                  80                  85

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Cys Phe Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly Lys Val
 90                  95                 100                 105

ttt caa gta cga aaa gta aca gga gca aat act ggg aaa ata ttt gcc   390
Phe Gln Val Arg Lys Val Thr Gly Ala Asn Thr Gly Lys Ile Phe Ala
                110                 115                 120

atg aag gtg ctt aaa aag gca atg ata gta aga aat gct aaa gat aca   438
Met Lys Val Leu Lys Lys Ala Met Ile Val Arg Asn Ala Lys Asp Thr
            125                 130                 135

gct cat aca aaa gca gaa cgg aat att ctg gag gaa gta aag cat ccc   486
Ala His Thr Lys Ala Glu Arg Asn Ile Leu Glu Glu Val Lys His Pro
        140                 145                 150

ttc atc gtg gat tta att tat gcc ttt cag act ggt gga aaa ctc tac   534
Phe Ile Val Asp Leu Ile Tyr Ala Phe Gln Thr Gly Gly Lys Leu Tyr
    155                 160                 165

ctc atc ctt gag tat ctc agt gga gga gaa cta ttt atg cag tta gaa   582
Leu Ile Leu Glu Tyr Leu Ser Gly Gly Glu Leu Phe Met Gln Leu Glu
170                 175                 180                 185

aga gag gga ata ttt atg gaa gac act gcc tgc ttt tac ttg gca gaa   630
Arg Glu Gly Ile Phe Met Glu Asp Thr Ala Cys Phe Tyr Leu Ala Glu
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            205                 210                 215

gac ctg aag ccg gag aat atc atg ctt aat cac caa ggt cat gtg aaa   726
Asp Leu Lys Pro Glu Asn Ile Met Leu Asn His Gln Gly His Val Lys
        220                 225                 230

cta aca gac ttt gga cta tgc aaa gaa tct att cat gat gga aca gtc   774
Leu Thr Asp Phe Gly Leu Cys Lys Glu Ser Ile His Asp Gly Thr Val
    235                 240                 245

aca cac aca ttt tgt gga aca ata gaa tac atg gcc cct gaa atc ttg   822
Thr His Thr Phe Cys Gly Thr Ile Glu Tyr Met Ala Pro Glu Ile Leu
250                 255                 260                 265

atg aga agt ggc cac aat cgt gct gtg gat tgg tgg agt ttg gga gca   870
Met Arg Ser Gly His Asn Arg Ala Val Asp Trp Trp Ser Leu Gly Ala
                270                 275                 280

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Leu Met Tyr Asp Met Leu Thr Gly Ala Pro Pro Phe Thr Gly Glu Asn
            285                 290                 295

aga aag aaa aca att gac aaa atc ctc aaa tgt aaa ctc aat ttg cct   966
Arg Lys Lys Thr Ile Asp Lys Ile Leu Lys Cys Lys Leu Asn Leu Pro
        300                 305                 310

ccc tac ctc aca caa gaa gcc aga gat ctg ctt aaa aag ctg ctg aaa   1014
Pro Tyr Leu Thr Gln Glu Ala Arg Asp Leu Leu Lys Lys Leu Leu Lys
    315                 320                 325

aga aat gct gct tct cgt ctg gga gct ggt cct ggg gac gct gga gaa   1062
Arg Asn Ala Ala Ser Arg Leu Gly Ala Gly Pro Gly Asp Ala Gly Glu
330                 335                 340                 345

gtt caa gct cat cca ttc ttt aga cac att aac tgg gaa gaa ctt ctg   1110
Val Gln Ala His Pro Phe Phe Arg His Ile Asn Trp Glu Glu Leu Leu
                350                 355                 360

gct cga aag gtg gag ccc ccc ttt aaa cct ctg ttg caa tct gaa gag   1158
Ala Arg Lys Val Glu Pro Pro Phe Lys Pro Leu Leu Gln Ser Glu Glu
            365                 370                 375

gat gta agt cag ttt gat tcc aag ttt aca cgt cag aca cct gtc gac   1206
Asp Val Ser Gln Phe Asp Ser Lys Phe Thr Arg Gln Thr Pro Val Asp
        380                 385                 390

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Ser Pro Asp Asp Ser Thr Leu Ser Glu Ser Ala Asn Gln Val Phe Leu
    395                 400                 405

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Gly Phe Thr Tyr Val Ala Pro Ser Val Leu Glu Ser Val Lys Glu Lys
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                430                 435                 440

cca cga aca cct gtc agc cca gtc aaa ttt tct cct ggg gat ttc tgg   1398
Pro Arg Thr Pro Val Ser Pro Val Lys Phe Ser Pro Gly Asp Phe Trp
            445                 450                 455

gga aga ggt gct tcg gcc agc aca gca aat cct cag aca cct gtg gaa   1446
Gly Arg Gly Ala Ser Ala Ser Thr Ala Asn Pro Gln Thr Pro Val Glu
        460                 465                 470

tac cca atg gaa aca agt ggc ata gag cag atg gat gtg aca atg agt   1494
Tyr Pro Met Glu Thr Ser Gly Ile Glu Gln Met Asp Val Thr Met Ser
    475                 480                 485

ggg gaa gca tcg gca cca ctt cca ata cga cag ccg aac tct ggg cca   1542
Gly Glu Ala Ser Ala Pro Leu Pro Ile Arg Gln Pro Asn Ser Gly Pro
490                 495                 500                 505

tac aaa aaa caa gct ttt ccc atg atc tcc aaa cgg cca gag cac ctg   1590
Tyr Lys Lys Gln Ala Phe Pro Met Ile Ser Lys Arg Pro Glu His Leu
                510                 515                 520

cgt atg aat cta tgacagagca atgcttttaa tgaatttaag gcaaaaaggt       1642
Arg Met Asn Leu
            525

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acatcaatta attgattcct cgcgacatct ttctcaacct tatcaaggat tttcatgttg 1882

atgactcgaa actgacagta ttaagggtag gatgttgctc tgaatcactg tgagtctgat 1942

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aggacaaatt agcatgcaag cttggtcaaa cttttcccag gcaaaatggg aaggcaaaga 2062

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<400> 4

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             20                  25                  30

Asp Gln Pro Glu Asp Ala Gly Ser Glu Asp Glu Leu Glu Glu Gly Gly
         35                  40                  45

Gln Leu Asn Glu Ser Met Asp His Gly Gly Val Gly Pro Tyr Glu Leu
     50                  55                  60

Gly Met Glu His Cys Glu Lys Phe Glu Ile Ser Glu Thr Ser Val Asn
 65                  70                  75                  80

Arg Gly Pro Glu Lys Ile Arg Pro Glu Cys Phe Glu Leu Leu Arg Val
                 85                  90                  95

Leu Gly Lys Gly Gly Tyr Gly Lys Val Phe Gln Val Arg Lys Val Thr
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Gly Ala Asn Thr Gly Lys Ile Phe Ala Met Lys Val Leu Lys Lys Ala
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Asn Ile Leu Glu Glu Val Lys His Pro Phe Ile Val Asp Leu Ile Tyr
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Ala Phe Gln Thr Gly Gly Lys Leu Tyr Leu Ile Leu Glu Tyr Leu Ser
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Gly Gly Glu Leu Phe Met Gln Leu Glu Arg Glu Gly Ile Phe Met Glu
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Asp Thr Ala Cys Phe Tyr Leu Ala Glu Ile Ser Met Ala Leu Gly His
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Leu His Gln Lys Gly Ile Ile Tyr Arg Asp Leu Lys Pro Glu Asn Ile
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Met Leu Asn His Gln Gly His Val Lys Leu Thr Asp Phe Gly Leu Cys
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Lys Glu Ser Ile His Asp Gly Thr Val Thr His Thr Phe Cys Gly Thr
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305                 310                 315                 320

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                325                 330                 335

Gly Ala Gly Pro Gly Asp Ala Gly Glu Val Gln Ala His Pro Phe Phe
            340                 345                 350

Arg His Ile Asn Trp Glu Glu Leu Leu Ala Arg Lys Val Glu Pro Pro
        355                 360                 365

Phe Lys Pro Leu Leu Gln Ser Glu Glu Asp Val Ser Gln Phe Asp Ser
    370                 375                 380

Lys Phe Thr Arg Gln Thr Pro Val Asp Ser Pro Asp Asp Ser Thr Leu
385                 390                 395                 400

Ser Glu Ser Ala Asn Gln Val Phe Leu Gly Phe Thr Tyr Val Ala Pro
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Ser Val Leu Glu Ser Val Lys Glu Lys Phe Ser Phe Glu Pro Lys Ile
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Arg Ser Pro Arg Arg Phe Ile Gly Ser Pro Arg Thr Pro Val Ser Pro
        435                 440                 445

Val Lys Phe Ser Pro Gly Asp Phe Trp Gly Arg Gly Ala Ser Ala Ser
    450                 455                 460

Thr Ala Asn Pro Gln Thr Pro Val Glu Tyr Pro Met Glu Thr Ser Gly
465                 470                 475                 480

Ile Glu Gln Met Asp Val Thr Met Ser Gly Glu Ala Ser Ala Pro Leu
                485                 490                 495

Pro Ile Arg Gln Pro Asn Ser Gly Pro Tyr Lys Lys Gln Ala Phe Pro
            500                 505                 510

Met Ile Ser Lys Arg Pro Glu His Leu Arg Met Asn Leu
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Ser Gln Lys

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<210> 8
<211> 18
<212> PRT
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<220>
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<400> 8
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1 5 10 15

Gln Lys

INTERNATIONAL SEARCH REPORT

Sonal Application No
PCT/US 99/17595

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/62 C12N9/12 C12N5/10 C07K16/40
G01N33/50 G01N33/566 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GOUT I. ET AL.: "Molecular cloning and characterization of a novel p70 S6 kinase beta containing a proline-rich region" J. BIOL. CHEM., vol. 273, no. 46, 13 November 1998 (1998-11-13), pages 30061-30064, XP002124654 the whole document	1-36
P,X	SAITOH M. ET AL.: "Cloning and characterization of p70(S6 beta) defines a novel family of p70 S6 kinases" BIOCHEM. BIOPHYS. RES. COMMUN., vol. 253, no. 2, 18 December 1998 (1998-12-18), pages 471-476, XP002124655 the whole document	1-36

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1999	11/01/2000

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 24463 A (INCYTE PHARMA INC ;MATHUR PREETE (US); REDDY ROOPA (US); AU YOUNG) 20 May 1999 (1999-05-20) compare nt 180-1450 of seq. ID 10 and nt 101-1370 of seq. ID 1 of present application	1-4
A	WO 98 18935 A (CIBA GEIGY AG ;THOMAS GEORGE (FR); KOZMA SARA (FR)) 7 May 1998 (1998-05-07) abstract claims 1-11	1-36
A	WO 93 19752 A (DANA FARBER CANCER INST INC) 14 October 1993 (1993-10-14) abstract	1-36
A	MUKHOPADHYAY N.K. ET AL.: "An array of insulin-activated, proline-directed serine/threonine kinases phosphorylate the p70 S6 kinase" J. BIOL. CHEM., vol. 267, no. 5, 15 February 1995 (1995-02-15), pages 3325-3335, XP002124656 the whole document	1-36
A	WENG Q.P. ET AL.: "Regulation of the p70 S6 kinase by phosphorylation in vivo" J. BIOL. CHEM., vol. 273, no. 26, 26 June 1998 (1998-06-26), pages 16621-16629, XP002124657 the whole document	1-36
A	ALESSI D.R. ET AL.: "3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro" CURRENT BIOLOGY, vol. 8, 10 December 1997 (1997-12-10), pages 69-81, XP000857264 the whole document	1-36

-/—

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAN J -W ET AL: "RAPAMYCIN, WORTMANNIN, AND THE METHYLXANTHINE SQ20006 INACTIVATE P70S6K BY INDUCING DEPHOSPHORYLATION OF THE SAME SUBSET OF SITES" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 270, no. 36, page 21396-21403 XP002057557 ISSN: 0021-9258 the whole document	1-36
A	PEARSON R B ET AL: "THE PRINCIPAL TARGET OF RAPAMYCIN-INDUCED P70S6K INACTIVATION IS A NOVEL PHOSPHORYLATION SITE WITHIN A CONSERVED HYDROPHOBIC DOMAIN" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 14, no. 21, page 5279-5287 XP000561164 ISSN: 0261-4189 the whole document	1-36
A	DENNIS P B ET AL: "THE PRINCIPAL RAPAMYCIN-SENSITIVE P70S6K PHOSPHORYLATION SITES, T-229 AND T-389, ARE DIFFERENTIALLY REGULATED BY RAPAMYCIN-INSENSITIVE KINASE KINASES" MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 16, no. 11, page 6242-6251 XP002057559 ISSN: 0270-7306 the whole document	1-36
A	WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA () 29 January 1998 (1998-01-29) abstract claims 1-9	1-36
A	PROUD C G: "P70 S6 KINASE: AN ENIGMA WITH VARIATIONS" TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 21, no. 5, page 181-185 XP002057556 ISSN: 0968-0004 the whole document	1-36

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/17595

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 10-11, as far as methods in vivo are concerned are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

See FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims 10-11 refer to modulating compounds, and claims 25-26 refer to binding partners of p70 S6K (beta) without giving a true technical characterization. Moreover, no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/17595

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9924463 A	20-05-1999	US 5932445 A	03-08-1999
		AU 1309599 A	31-05-1999
WO 9818935 A	07-05-1998	AU 5314598 A	22-05-1998
		EP 0942990 A	22-09-1999
WO 9319752 A	14-10-1993	AU 3922493 A	08-11-1993
WO 9803662 A	29-01-1998	AU 4113897 A	10-02-1998
		EP 0915982 A	19-05-1999

CORRECTED VERSION*

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/54, 15/62, 9/12, 5/10, C07K 16/40, G01N 33/50, 33/566, C12Q 1/68

A1

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(22) International Filing Date: 4 August 1999 (04.08.99)

(30) Priority Data:
60/095,268 4 August 1998 (04.08.98) US

(71) Applicant *(for all designated States except US)*: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 34th floor, 1345 Avenue of the Americas, New York, NY 10105 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GOUT, Ivan [–/US]; Ludwig Institute for Cancer Research, 34th floor, 1345 Avenue of the Americas, New York, NY 10105 (US). HARA, Kenta [–/US]; Ludwig Institute for Cancer Research, 34th floor, 1345 Avenue of the Americas, New York, NY 10105 (US). WATERFIELD, Mike [–/US]; Ludwig Institue for Cancer Research, 34th floor, 1345 Avenue of the Americas, New York, NY 10105 (US). YONEZAWA, Kazu [–/US]; Ludwig Institute for Cancer Research, 34th floor, 1345 Avenue of the Americas, New York, NY 10105 (US).

(74) Agent: ADLER, Reid, G.; Morgan, Lewis & Bockius LLP, 1800 M Street, N.W., Washington, DC 20236–5869 (US).

(81) Designated States: AU, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL RIBOSOMAL S6 PROTEIN KINASE

(57) Abstract

A novel S6 kinase, p70β^{S6k}, is described, along with methods of making and using p70β^{S6k} and related nucleic acids. The invention also discloses methods of identifying agents which modulate the activity of p70β^{S6k} and/or its ligands.

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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL RIBOSOMAL S6 PROTEIN KINASE

This invention relates to United States Provisional Application Serial No. 60/095,268, filed August 4, 1988, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention relates to a novel S6 kinase ($p70\beta^{Sk6}$), mutant variants thereof, methods of making and using this S6 kinase, and related nucleic acids and antibodies. The invention also relates to binding partners of the S6 kinase, methods of identifying the binding partners and antibodies thereto.

BACKGROUND OF THE INVENTION

The 40S ribosomal protein S6 is a component of the 40S subunit of eukaryotic ribosomes. The ribosomes are part of the cellular machinery responsible for translation of mRNA and protein synthesis. The S6 protein is phosphorylated in response to certain cellular signaling events such as hormone or growth factor induced cellular proliferation. p70 S6 kinase ($p70^{S6k}$) is responsible for S6 phosphorylation and is believed to be the major physiological S6 kinase in mammalian cells (Proud, 1996 Trends Biochem. Sci. 21: 181-185).

1. p70α S6 Kinase

A. Structure and Function

The first p70 S6 kinase identified was the alpha (α) form. The gene encoding the human p70α S6 kinase ($p70^{S6k}$)was isolated in 1991 (Grove *et al.*, 1991 Mol. Cell.

Biol. 11: 5541-5550). Other p70α S6 kinase sequences have been described in *Mus musculus* (GenBank Accession No. SEG_AB015196S, AB015197, and AB015196), *Xenopus laevis* (GenBank Accession No. X66179), and rat (GenBank Accession No. M57428).

Two p70α S6 kinase isoforms were identified: p70α-I (GenBank Accession No. M60724) and p70α-II (GenBank Accession No. M60725). The two p70α S6 kinase isoforms differ only in their amino termini by 23 amino acid residues resulting in a 70 kD protein and a 85 kD protein. The isoforms are referred to in the literature as $p70^{S6k}/p85^{S6k}$ or p70α S6 kinase. Both isoforms share similar activity towards ribosomal protein S6 *in vitro* but are expressed in different cells and tissues. The two isoforms are produced by two mRNA products and are not a result of post-translational modifications. They are serine/threonine kinases and are known to act on the substrate KKRNRTLSVA (SEQ ID No. 7) (Pai *et al.*, 1994 Eur. J. Immunol. 24: 2364-8; and Leighton *et al.*, 1995 FEBS Letters 375: 289-93).

The p70α S6 kinase plays an important role in the progression of cells from G1 to S phase of the cell cycle and in the initiation of protein synthesis. Recently, p70α S6 kinase has been demonstrated to regulate the translation of a class of mRNAs containing an oligopyrimidine tract in their 5' untranslated region. This class of mRNAs, termed 5'TOP mRNAs, represent up to 20% of the a cell's total mRNA. Many of the proteins encoded by 5'TOP mRNAs are translational apparatus proteins and cell-cycle progression proteins.

The p70α S6 kinase has four identified interdependent domains: (1) a catalytic domain, (2) a kinase extension domain, (3) a pseudosubstrate autoinhibitory domain, and (4) the N-terminal domain. The catalytic domain is located in the middle of the protein and is followed by the kinase extension domain, which is a unique feature for the PKA family. The pseudosubstrate autoinhibitory domain is also unique for the p70α S6 kinase, not having been observed in any other known kinases. It possesses 5 phosphorylation sites which are responsible for the p70α S6 kinase regulation. The N-terminal domain mediates the sensitivity for rapamycin, which strongly inhibits serum-

induced phosphorylation and activation of the p70α S6 kinase. This domain may also mediate the interaction with a yet unknown phosphatase.

B. Regulators and Cascades

Growth factors, such as insulin, and mitogens are known to activate *in vivo* p70α S6 kinase (Alessi *et al.*, 1998 Curr. Biol. 8: 69-81). Heat shock also activates p70α S6 kinase (Lin *et al.*, 1997 J. Biol. Chem. 272: 31196-31202). Certain drugs have been identified that regulate p70α S6 kinase activity including: rapamycin, wortmannin, Ro31-8220, GF109203X, LY294002, phenylephrine (PE), PD098059, SQ20006, polymerized collagen, forskolin, interleukin-10 (IL-10), demethoxyviridin, phorbol 12-myristate 13-acetate (PMA), A23187, bombesin and antibodies which recognize the p70α S6 kinase (Proud, 1996; Morreale *et al.*, 1997 FEBS Letters 417: 38-42; Kanda *et al.*, 1997 J. Biol. Chem. 272: 23347-23353; Boluyt *et al.*, 1997 Circ. Res. 81: 176-186; Coolican *et al.*, 1997 J. Biol. Chem. 272: 6653-6662; Koyama *et al.*, 1996 Cell 87: 1069-1078; Busca *et al.*, 1996 J. Biol. Chem. 271: 31824-31830; Crawley *et al.*, 1996 J. Biol. Chem. 271: 16357-16362; and Petritsch *et al.*, 1995 Eur. J. Biochem. 230: 431-8). The immunosuppressant rapamycin (Rap) is the most potent inhibitor of p70α S6 kinase described (Pullen *et al.*, 1997 FEBS Letters 410: 78-82).

p70α S6 kinase is an enzyme which lies downstream of phosphoinositide 3-kinases (PI3-kinase). The mechanisms regulating the p70α S6 kinase have not been fully elucidated. PI3-kinase has recently been shown to activate another phosphoinositide-dependent protein kinase, termed PDK-1. So far, only PDK-1 has been shown to phosphorylate p70α S6 kinase *in vivo*, and this phosphorylation is essential for $p70\alpha^{S6k}$ activity towards ribosomal S6 protein. Wortmannin, a fungal inhibitor which down-regulates the p70α S6 kinase, is believed to act by inhibiting PI-3 kinase. In contrast, another fungal inhibitor, rapamycin, inhibits the p70α S6 kinase by another cascade pathway involving the mammalian target of rapamycin (mTOR; also known as RAFT or FRAP) (Proud, 1996; Stewart *et al.*, 1994 BioEssays 16: 809-815). mTOR is a member of the PIK-related family of protein kinases (Pullen *et al.*, 1997).

Additional regulators of the p70α S6 kinase include, but are not limited to protein kinase B (PKB), Cdc42, and Rac. The role of most of these proteins as p70α S6 kinase regulators has yet to be fully elucidated.

SUMMARY OF THE INVENTION

The present invention is based on our discovery of a new gene which encodes a novel S6 kinase ($p70\beta^{S6k}$). The invention includes isolated nucleic acid molecules selected from the group consisting of an isolated nucleic acid molecule that encodes the amino acid sequence of SEQ ID No.2, (*e.g.*, SEQ ID No.1) an isolated nucleic acid molecule that encodes a fragment of SEQ ID No.2, an isolated nucleic acid molecule which hybridizes to the complement of a nucleic acid molecule comprising SEQ ID No.1 under conditions of sufficient stringency to produce a clear signal and an isolated nucleic acid molecule which hybridizes to the complement of a nucleic acid molecule that encodes the amino acid sequence of SEQ ID No.2 under conditions of sufficient stringency to produce a clear signal.

The present invention further includes the nucleic acid molecules operably linked to one or more expression control elements, including vectors comprising the isolated nucleic acid molecules. The invention further includes host cells transformed to contain the nucleic acid molecules of the invention and methods for producing a protein comprising the step of culturing a host cell transformed with the nucleic acid molecule of the invention under conditions in which the protein is expressed.

The invention further provides an isolated polypeptide selected from the group consisting of an isolated polypeptide comprising the amino acid sequence of SEQ ID No.2, an isolated polypeptide comprising a fragment of SEQ ID No.2, an isolated polypeptide comprising conservative amino acid substitutions of SEQ ID No.2 and naturally occurring amino acid sequence variants of SEQ ID No.2.

The invention further provides an isolated antibody that binds to a polypeptide of the invention, including monoclonal and polyclonal antibodies and fragments

thereof.

The invention further provides methods of identifying an agent which modulates the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2 comprising the steps of: exposing cells which express the nucleic acid to the agent; and determining whether the agent modulates expression of said nucleic acid, thereby identifying an agent which modulates the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2.

The invention further provides methods of identifying an agent which modulates at least one activity of a protein comprising the sequence of SEQ ID No.2 comprising the steps of: exposing cells which express the protein to the agent; and determining whether the agent modulates at least on activity of said protein, thereby identifying an agent which modulates at least one activity of a protein comprising the sequence of SEQ ID No.2.

The invention further provides methods of identifying binding partners for a protein comprising the sequence of SEQ ID No.2 or activated variants thereof, comprising for example, the steps of: exposing said protein to a potential binding partner; and determining if the potential binding partner binds to said protein, thereby identifying binding partners for a protein comprising the sequence of SEQ ID No.2. Exposing may be accomplished by expressing the protein in a cell.

The present invention further provides methods of modulating the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2 comprising the step of: administering an effective amount of an agent which modulates the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2. The invention also provides methods of modulating at least one activity of a protein comprising the sequence of SEQ ID No.2 comprising the step of: administering an effective amount of an agent which modulates at least one activity of a protein comprising the sequence of SEQ ID No.2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleic acid sequence of p70β^{S6k} (SEQ ID No.1) and comparison with p70α^{S6k} (SEQ ID No. 3). Sequence analysis of cDNA encoding the p70β S6 kinase. p70β cDNA may encode two isoforms with the use of alternative start codon. The longer form may encode a protein of 495 amino acid residues and the shorter form, 482 amino acids (aa). Alternative start codons and a stop codon are highlighted.

Figure 2A-2B. Amino Acid Sequence of p70β^{S6k} (SEQ ID No.2) and comparison with p70α^{S6K} (SEQ ID No. 4).

Figure 2A. Alignment of predicted protein sequences corresponding to the p70α^{S6K} and p70β S6 kinases. Identical amino acids are boxed.

Figure 2B. Comparative analysis of regulatory domains and phosphorylation sites between p70α and β S6 kinases.

Figure 3A - 3B. Tissue Specific Expression of p70β^{S6k}. Northern blot analysis of poly(A)+ RNA from human tissues (Figure 3A) and tumor cell lines (Figure 3B).

Figure 3A. Nylon membrane containing 2 μg of gel-separated, pre-bound poly(A)+ RNA samples from various human tissues was hybridized with cDNA fragments of p70α^{S6k}, p70β^{S6k} or β-actin labeled by random priming. The upper, middle and lower panels are autoradiographs probed with p70β^{S6k}, p70α^{S6k} and β-actin, respectively. Each lane contains mRNA prepared from: heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), pancreas (lane 8), spleen (lane 9), thymus (lane 10), prostate (lane 11), testis (lane 12), ovary (lane 13), small intestine (lane 14), mucosal lining of the colon (lane 15), and peripheral blood leukocytes (lane 16).

Figure 3B. Nylon membrane containing 2 μg of mRNA isolated from tumor cell lines was probed with the 3' cDNA fragment from p70β^{S6k}, which was labeled by random-prime labeling. Specific binding was determined by autoradiography. Promyelocytic leukemia HL-60 (lane 1), HeLa cell S3 (lane 2, chronic myelogenous leukemia K562 (lane 3), lymphoblastic leukemia MOLT-4 (lane 4), Burkitt's

lymphoma Raji (lane 5), colorectal adenocarcinoma SW480 (lane 6), lung carcinoma (lane 7), and melanoma G361 (lane 8).

Figure 4A - 4B. Phosphorylation of the ribosomal protein S6 (Figure 4A) and its C-terminal synthetic peptide (Figure 4B) by $p70\alpha^{S6k}$ and $p70\beta^{S6k}$. Ribosomal S6 protein (purified ribosomal 40S subunits from liver) and synthetic peptides corresponding to the S6 protein C-terminus (*e.g.*, KEAKEKRQEQIARRRLSSLRASTSKSESSQK-long form (SEQ ID No. 5) and RRRLSSLRASTSKSESSQK-(SEQ ID No. 6) short form) were used to measure the activity of the $p70\alpha^{S6k}$ and $p70\beta^{S6k}$. HEK293 cells were transfected with plasmids containing Flag-tag or EE-tag versions of $p70\alpha^{S6k}$ or $p70\beta^{S6k}$. Recombinant proteins were immunoprecipitated with anti-EE or anti-Flag antibodies and an *in vitro* kinase reaction performed in the presence of the ribosomal 40S subunits or synthetic peptides. After SDS-PAGE analysis, phosphorylation of the S6 protein and synthetic peptides was measured by PhosphoImager and expressed in arbitrary units (PI units).

Figure 5A - 5B. Stimulation of $p70\beta^{S6k}$ Activity by insulin, serum and TPA.

Figure 5A. CHO-IR cells were transfected with mock (lane1) or plasmids containing cDNAs of FLAG-tagged p70α-I (lanes 2 and 3) or FLAG-tagged p70β-II (lanes 4 to 7). After serum starvation for 16 hrs, cells were treated with the vehicle (lanes 2 and 4), 10^{-7} M insulin for 10 min (lanes 3 and 5), 15% serum for 10 min (lane 6) or 500 nM TPA for 30 min (lane7). After cell lysis and subsequent immunoprecipitation with anti-FLAG antibodies, immunoprecipitates were subjected to a p70 S6 kinase assay using 40S subunits as substrates. The reaction mixture was separated by SDS-PAGE, transferred onto PVDF membrane. The membrane was analyzed by autoradiography (upper panel) and then immunoblotted with anti-FLAG antibodies (lower panel). A representative of three experiments is shown. ^{32}P incorporation into S6 was quantified by Molecular Dynamics PhosphorImager™ and is expressed in arbitrary units (PI units).

Figure 5B. Stimulation of the p70α^{S6k} and p70β^{S6k} activity towards ribosomal S6 protein by PDGF in PAE-PDGF-R cells. PAE-PDGF-R cells were transfected with EE-tagged p70α^{S6k} and p70β^{S6k} plasmids using lipofectAMINE. After 24 hr, transfected cells were serum-starved for 16 hr and stimulated with 20 ng/ml PDGF BB (Calbiochem) for 20 min. Control cells were treated with the vehicle under the same conditions. After immunoprecipitation with anti-EE antibodies, an *in vitro* kinase reaction was carried out in the presence of 40S subunits, containing the S6 protein. Reaction mixtures were separated by SDS-PAGE and incorporation of ^{32}P into S6 protein was measured by PhosphoImager.

Figure 6A - 6B. Effects of rapamycin and wortmannin on p70β^{S6k} and p70α^{S6k}. Effects of rapamycin and wortmannin on S6 phosphorylation activity of p70α-I or p70β-II. HEK293 cells were transfected with mock cDNA or plasmids containing FLAG-tagged p70α1 or p70β2 isoforms. After 48 h of transfection, cells were treated with vehicle or indicated concentrations of rapamycin or wortmannin for 30 min. After immunoprecipitation with anti-FLAG antibodies, the kinase activity was determined by a p70 S6 kinase assay using 40S subunits as substrates. The proteins of the reaction mixture were separated by SDS-PAGE, transferred onto PVDF membrane and analyzed by autoradiography (Figures 6A and 6B, upper panels). Subsequent immunoblotting with anti-FLAG antibody confirmed the expression of p70α-I (Figure 6A, lower panel) and p70β-II (Figure 6B, lower panel). A representative of three experiments is shown. ^{32}P incorporation into S6 was quantified by PhosphorImager and is expressed in arbitrary units (PI units).

Figure 7. Interaction of the p70β^{S6k} with different GST/SH3 fusion proteins. HEK293 cells were transiently transfected with EE-tag/p70β^{S6k}. Two days later, cells were lysed and the lysates were immunoprecipitated with anti-EE antibodies. GST/SH3 fusion proteins (1.5 μg each) were incubated with anti-EE tag immunoprecipitates. Specific interaction with p70β^{S6k} was measured by anti-GST

immunoblotting. SH3 domains from different signaling and cytoskeletal proteins were expressed in bacteria as GST fusion proteins and purified nearly to homogeneity using glutathione-Sepharose beads. The GST/SH3 fusion proteins used are: GST (lane 1), p80α subunit of the PI3-kinase (lane 2), GAP (lane 3), PLCγ (lane 4), spectrin (lane 5), crk (lane 6), n-grb2 (lane 7), c-grb2 (lane 8), grb2 full (lane 9), csk (lane 10), fgr (lane 11), fyn (lane 12), src (lane 13), ruk a (lane 14), ruk b (lane 15), ruk c (lane 16), p15 (lane 17), profilin (lane 18) and GST/GAP control (lane 19). "↑" indicates instances of binding between $p70\beta^{S6k}$ and a SH3 containing fusion protein.

Figure 8. Immunoprecipitation and Western blot analysis of the p70β-I and p70β-II isoforms transiently over expressed in HEK293 cells with anti-$p70\beta^{S6k}$ antibodies. The lanes are the same for each panel: mock transfected (lane 1), Flag-p70α-I transfected (lane 2), Flag-p70β-I transfected (lane 3), and Flag-p70β-II transfected (lane 4).

Figure 9. A model for the activation of $p70^{S6}$ Kinase. Schematic presentation of the $p70\alpha^{S6k}$ structure, protein-protein interactions, activation levels and phosphorylation state.

Figure 10. $p70\beta^{S6k}$ mutations. Schematic presentation of substitution mutations engineered into $p70\beta^{S6k}$, including a change of Threonine at amino acid 401 to Aspartic acid (T401D).

Figure 11. $p70\beta^{S6k}$ (T401D) activity. Activity of $p70\beta^{S6k}$ (T401D) variant as compared to wt $p70\beta^{S6k}$ under S6 Kinase and autophosphorylation assays.

Figure 12: $p70\alpha^{S6k}$ (T412D) activity. Activity of $p70\alpha^{S6k}$ (T412D) variant as compared to wt $p70\alpha^{S6k}$ under S6 Kinase and autophosphorylation assays.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The terms "p70α", "p70α^{S6k}" and "p70α ^{S6} kinase" are meant to include the two isoforms, p70 and p85, both of which phosphorylate the ribosomal protein S6. By "p70α-1" and "p70α-I" are meant the p85 isoform of the p70α ^{S6} kinase. By "p70α-2" and "p70α-II" are meant the p70 isoform of the p70α ^{S6} kinase.

The terms "p70β", "p70β^{S6k}" and "p70β ^{S6} kinase" include the newly identified S6 kinase and all its isoforms.

I. General Description

The present invention is based in part on identifying a new gene that encodes a novel S6 kinase (p70β^{S6k}). This new gene and the protein that it encodes are members of the family of S6 kinases, of which the p70α-I and -II (also referred to as p70α-1 and p70α-2) isoforms have already been reported.

The protein can serve as a target for agents that can be used to modulate the expression or activity of the protein. For example, agents may be identified which modulate biological processes associated with ribosomal activity.

The present invention is further based on the development of methods for isolating binding partners that bind to the protein or its activated variants. Probes based on the protein are used as capture probes to isolate potential binding partners, such as other proteins. Dominant negative proteins, DNAs encoding these proteins, antibodies to these proteins, peptide fragments of these proteins or mimics of these proteins may be introduced into cells to affect function. Additionally, these proteins provide a novel target for screening of synthetic small molecules and combinatorial or naturally occurring compound libraries to discover novel therapeutics to regulate ribosomal function.

II. Specific Embodiments

A. The Ribosome Associated Protein

The present invention provides isolated protein, allelic variants of the protein, and conservative amino acid substitutions of the protein, including substitutions that activate the protein. As used herein, the protein or polypeptide refers to a protein that has the human amino acid sequence of depicted in SEQ ID No.2. The invention includes naturally occurring allelic variants and proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the same or similar biological functions associated with the disclosed protein.

As used herein, the family of proteins related to the disclosed protein refer to proteins that have been isolated from organisms in addition to humans. The methods used to identify and isolate other members of the family of proteins related to the disclosed protein are described below.

The proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated protein.

The proteins of the present invention further include conservative variants of the proteins herein described. As used herein, a conservative variant refers to alterations in the amino acid sequence that do not adversely affect the biological functions of the protein. A substitution, insertion or deletion is said to adversely affect the protein when the altered sequence prevents or disrupts a biological function associated with the protein. For example, the overall charge, structure or hydrophobic/hydrophilic properties of the protein can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the biological activities of the protein. Conservative substitutions may irreversibly activate the protein.

Ordinarily, the allelic variants, the conservative substitution variants, the members of the protein family, will have an amino acid sequence having at least 71%-about 75% amino acid sequence identity with the human sequence set forth in SEQ ID No.2, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Homology or identity is determined by **BLAST** (**B**asic **L**ocal **A**lignment **S**earch **T**ool) analysis using the algorithm employed by the programs **blastp**, **blastn**, **blastx**, **tblastn** and **tblastx** (Karlin , et al. Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990) and Altschul, S. F. J. Mol. Evol. 36: 290-300(1993), fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al. (Nature Genetics 6: 119-129 (1994)) which is fully incorporated by reference. The search parameters for **histogram**, **descriptions**, **alignments**, **expect** (i.e., the statistical significance threshold for reporting matches against database sequences), **cutoff**, **matrix** and **filter** are at the default settings. The default scoring matrix used by **blastp**, **blastx**, **tblastn**, and **tblastx** is the **BLOSUM62** matrix (Henikoff, et al. Proc. Natl. Acad. Sci. USA 89: 10915-10919 (1992), fully incorporated by reference). For **blastn**, the scoring matrix is set by the ratios of **M** (i.e., the reward score for a pair of matching residues) to **N** USA 87: 2264-2268 (1990) and (i.e., the penalty score for mismatching residues), wherein the default values for **M** and

N are 5 and -4, respectively.

Thus, the proteins of the present invention include molecules having the amino acid sequence disclosed in SEQ ID No.2; fragments thereof having a consecutive sequence of at least about 3, 5, 10 or 15 amino acid residues of the disclosed protein; amino acid sequence variants of such sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, the disclosed sequence; amino acid sequence variants of the disclosed sequence, or their fragments as defined above, that have been substituted by another residue. Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding proteins of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the family of proteins; and derivatives wherein the protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope). Proteins of the invention may include fusion proteins comprising any of the foregoing.

As described below, members of the family of proteins can be used: 1) to identify agents which modulate at least one activity of the protein, including agents which may modulate phosphorylation mediated by the protein; 2) in methods of identifying binding partners for the protein, 3) as an antigen to raise polyclonal or monoclonal antibodies, and 4) as a therapeutic agent.

B. Nucleic Acid Molecules

The present invention further provides nucleic acid molecules that encode the protein having SEQ ID No.2 and the related proteins herein described, preferably in isolated form. As used herein, "nucleic acid" is defined as RNA or DNA that encodes a peptide as defined above, or is complementary to nucleic acid sequence encoding such peptides, or hybridizes to such nucleic acid and remains stably bound to it under

appropriate stringency conditions, or encodes a polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85%, with the peptide sequences. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such hybridizing or complementary nucleic acids, however, are defined further as being novel and nonobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to nucleic acid encoding a protein according to the present invention.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl, 0.0015 M sodium titrate, 0.1% SDS at 50°C; or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid.

The present invention further provides fragments of the encoding nucleic acid molecule. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the protein, the fragment will need to be large enough to encode the

functional region(s) of the protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the encoding nucleic acid molecules of the present invention (i.e., synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding proteins of the invention can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, (J. Am. Chem. Soc. 103: 3185-3191, 1981) or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can employ any of the art known labels to obtain a labeled encoding nucleic acid molecule.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

C. Isolation of Other Related Nucleic Acid Molecules

As described above, the identification of the human nucleic acid molecule having SEQ ID No.1 allows a skilled artisan to isolate nucleic acid molecules that encode other members of the $p70\beta^{S6k}$ family in addition to the human sequence herein described. Further, the presently disclosed nucleic acid molecules allow a skilled artisan to isolate

nucleic acid molecules that encode other members of the $p70\beta^{S6k}$ family of proteins in addition to the disclosed protein having SEQ ID No.2.

Essentially, a skilled artisan can readily use the amino acid sequence of SEQ ID No.2 to generate antibody probes to screen expression libraries prepared from appropriate cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as *λgtll* library, to obtain the appropriate coding sequence for other members of the protein family. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme.

Alternatively, a portion of the coding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the protein family from any mammalian organism. Oligomers containing approximately 18-20 or 21 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively clone an encoding nucleic acid molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other encoding nucleic acid molecules.

D. rDNA molecules Containing a Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a coding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, (1989). In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the protein family encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the structural gene included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a coding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or bacteriophage promoter capable of directing the expression (transcription and translation) of the coding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form a rDNA molecules the contains a coding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene (Southern *et al.*, J. Mol. Anal. Genet. 1: 327-341, 1982). Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

E. Host Cells Containing an Exogenously Supplied Coding Nucleic Acid Molecule

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a protein of the invention are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), and the like eukaryotic

tissue culture cell lines.

Any prokaryotic host can be used to express a rDNA molecule encoding a protein of the invention. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, Proc. Natl. Acad. Sci. USA 69: 2110, 1972; and Maniatis *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, Virol. 52: 456, 1973; Wigler *et al.*, Proc. Natl. Acad. Sci. USA 76: 1373-76, 1979.

Successfully transformed cells, i.e., cells that contain a rDNA molecule of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, J. Mol. Biol. 98:503, 1975, or Berent *et al.*, Biotech. 3: 208, 1985 or the proteins produced from the cell assayed via an immunological method.

Recombinant $p70\beta^{S6k}$ DNA can also be utilized to analyze the function of coding and non-coding sequences. For example, the 5' untranslated region of the $p70\beta^{S6k}$ clone contains a GA repeat (nucleotides 1-66 of $P70\beta^{S6k}$), that may modulate the initiation of translation of its mRNA. This sequence can be utilized in an affinity matrix system to purify proteins obtained from cell lysates that associate with the $p70\beta^{S6k}$ GA sequence. Synthetic oligonucleotides would be coupled to the beads and probed with the lysates, as is commonly known in the art. Associated proteins could then be separated using, for example, a two dimensional SDS-PAGE system. Proteins thus isolated could be further

identified using mass spectroscopy or protein sequencing.

F. Production of Recombinant Proteins using a rDNA Molecule

The present invention further provides methods for producing a protein of the invention using nucleic acid molecules herein described. In general terms, the production of a recombinant form of a protein typically involves the following steps:

First, a nucleic acid molecule is obtained that encodes a protein of the invention, such as the nucleic acid molecule depicted in SEQ ID No. 1, or particularly for the $p70\beta^{S6k}$ nucleotides encoding the proline rich domain or the amino terminus of $p70\beta^{S6k}$. The coding sequence is directly suitable for expression in any host, as it is not interrupted by introns. The sequence can be transfected into host cells such as eukaryotic cells or prokaryotic cells. Eukaryotic hosts include mammalian cells (*e.g.*, HEK293 cells, CHO cells and PAE-PDGF-R cells) as well as insect cells such as Sf9 cells using recombinant baculovirus. Alternatively, fragments encoding only portion of $p70\beta^{S6k}$ can be expressed alone or in the form of a fusion protein. For example, the C-terminal fragment of $p70\beta^{S6k}$ containing the proline-rich domain, was expressed in bacteria as a GST- or His-tag fusion protein. These fusion proteins were then purified and used to generate polyclonal antibodies.

The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the protein open reading frame. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the recombinant protein. Optionally the recombinant protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with the nucleic acid molecules of the invention to produce recombinant protein.

G. In Vitro Methods to Identify Binding Partners

Another embodiment of the present invention provides methods for use in isolating and identifying binding partners of proteins of the invention In detail, a protein of the invention is mixed with a potential binding partner or an extract or fraction of a cell under conditions that allow the association of potential binding partners with the protein of the invention. After mixing, peptides, polypeptides, proteins or other molecules that have become associated with a protein of the invention are separated from the mixture. The binding partner that bound to the protein of the invention can then be removed and further analyzed. To identify and isolate a binding partner, the entire protein, for instance the entire disclosed protein of SEQ ID No.2 can be used. Alternatively, a fragment of the protein can be used.

As used herein, a cellular extract refers to a preparation or fraction which is made from a lysed or disrupted cell.

A variety of methods can be used to obtain cell extracts. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

Once an extract of a cell is prepared, the extract is mixed with the protein of the invention under conditions in which association of the protein with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that

closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract used, can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to a protein of the invention can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

To aid in separating associated binding partner pairs from the mixed extract, the protein of the invention can be immobilized on a solid support. For example, the protein can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the protein to a solid support aids in separating peptide/binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins.

Alternatively, the nucleic acid molecules of the invention can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the nucleic acid molecules herein described.

One preferred *in vitro* binding assay for $p70\beta^{S6k}$ would comprise a mixture of a polypeptide comprising at least the kinase domain of $p70\beta^{S6k}$ and one or more candidate binding targets or substrates. After incubating the mixture under appropriate conditions, one would determine whether $p70\beta^{S6k}$ or a polypeptide fragment thereof containing the kinase region either bound with the candidate substrate or phosphorylated the candidate substrate. For cell-free binding assays, one of the

components usually comprises or is coupled to a label. The label may provide for direct detection, such as radioactivity, luminescence, optical or electron density, *etc.*, or indirect detection such as an epitope tag, an enzyme, *etc.* A variety of methods may be employed to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and the label thereafter detected.

H. Methods to Identify Agents that Modulate the Expression a Nucleic Acid Encoding the S6 Kinase Protein.

Another embodiment of the present invention provides methods for identifying agents that modulate the expression of a nucleic acid encoding a protein of the invention such as a protein having the amino acid sequence of SEQ ID No.2. Such assays may utilize any available means of monitoring for changes in the expression level of the nucleic acids of the invention. As used herein, an agent is said to modulate the expression of a nucleic acid of the invention, for instance a nucleic acid encoding the protein having the sequence of SEQ ID No.2, if it is capable of up- or down-regulating expression of the nucleic acid in a cell.

In one assay format, cell lines that contain reporter gene fusions between the open reading frame defined by $p70\beta^{S6k}$ nucleotides 77-1,564 or 116-1,564 of SEQ ID No.1 and any assayable fusion partner may be prepared. Numerous assayable fusion partners are known and readily available including the firefly luciferase gene and the gene encoding chloramphenicol acetyltransferase (Alam *et al.*, 1990 Anal. Biochem. 188: 245-254). Cell lines containing the reporter gene fusions are then exposed to the agent to be tested under appropriate conditions and time. Differential expression of the reporter gene between samples exposed to the agent and control samples identifies agents which modulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2.

Additional assay formats may be used to monitor the ability of the agent to

modulate the expression of a nucleic acid encoding a protein of the invention such as the protein having SEQ ID No.2. For instance, mRNA expression may be monitored directly by hybridization to the nucleic acids of the invention. Cell lines are exposed to the agent to be tested under appropriate conditions and time and total RNA or mRNA is isolated by standard procedures such those disclosed in Sambrook *et al.* (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed. Cold Spring Harbor Laboratory Press, 1989).

Probes to detect differences in RNA expression levels between cells exposed to the agent and control cells may be prepared from the nucleic acids of the invention. It is preferable, but not necessary, to design probes which hybridize only with target nucleic acids under conditions of high stringency. Only highly complementary nucleic acid hybrids form under conditions of high stringency. Accordingly, the stringency of the assay conditions determines the amount of complementarity which should exist between two nucleic acid strands in order to form a hybrid. Stringency should be chosen to maximize the difference in stability between the probe:target hybrid and potential probe:non-target hybrids.

Probes may be designed from the nucleic acids of the invention through methods known in the art. For instance, the G+C content of the probe and the probe length can affect probe binding to its target sequence. Methods to optimize probe specificity are commonly available in Sambrook *et al.* (1989) or Ausubel *et al.* (CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Co., NY, 1995).

Hybridization conditions are modified using known methods, such as those described by Sambrook *et al.* (1989) and Ausubel *et al.* (1995) as required for each probe. Hybridization of total cellular RNA or RNA enriched for polyA RNA can be accomplished in any available format. For instance, total cellular RNA or RNA enriched for polyA RNA can be affixed to a solid support and the solid support exposed to at least one probe comprising at least one, or part of one of the sequences of the invention under conditions in which the probe will specifically hybridize. Alternatively, nucleic acid fragments comprising at least one, or part of one of the

sequences of the invention can be affixed to a solid support, such as a porous glass wafer. The glass wafer can then be exposed to total cellular RNA or polyA RNA from a sample under conditions in which the affixed sequences will specifically hybridize. Such glass wafers and hybridization methods are widely available, for example, those disclosed by Beattie (WO 95/11755). By examining for the ability of a given probe to specifically hybridize to an RNA sample from an untreated cell population and from a cell population exposed to the agent, agents which up or down regulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID No.2 are identified.

I. Cell-Based Methods to Identify Binding Partners and Agents that Modulate at Least One Activity of the S6 Kinase Protein and Related Antibodies.

Another embodiment of the present invention provides methods for identifying agents that modulate at least one activity of a protein of the invention such as the protein having the amino acid sequence of SEQ ID No.2. Such methods or assays may utilize any means of monitoring or detecting the desired activity.

In one format, the relative amounts of a protein of the invention between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein in the different cell populations. Cell lines or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysates may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysates are then analyzed with the probe.

For example, N- and C- terminal fragments of $p70\beta^{S6k}$ can be expressed in bacteria and used to search for proteins which bind to these fragments. Fusion proteins, such as His-tag or GST fusion to the N- or C-terminal regions of $p70\beta^{S6k}$ can be prepared for use as a $p70\beta^{S6k}$ fragment substrate. These fusion proteins can be coupled to Talon or Glutathione-Sepharose beads and then probed with cell lysates. Prior to

lysis, the cells may be treated with rapamycin or other drugs which may modulate $p70\beta^{S6k}$ or proteins that interact with $p70\beta^{S6k}$. Lysate proteins binding to the fusion proteins can be resolved by SDS-PAGE, isolated and identified by protein sequencing or mass spectroscopy, as is known in the art. It is likely that signaling molecules containing one or more SH3 domains may bind directly to the C-terminal region of $p70\beta^{S6k}$. The N-terminal domain may have a $p70\beta^{S6k}$-specific phosphatase as a binding partner.

Antibody probes are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptides, polypeptides or proteins of the invention, such as $p70\beta^{S6k}$, variants and isolated binding partners, if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a Cys residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

Anti-peptide antibodies can be generated using synthetic peptides corresponding to, for example, the carboxy terminal 15 amino acids $p70\beta^{S6k}$. Synthetic peptides can be as small as 1-3 amino acids in length, but are preferably at least 4 or more amino acid residues long. The peptides are coupled to KLH using standard methods and can be immunized into animals such as rabbits. Polyclonal anti-$p70\beta^{S6k}$ peptide antibodies can then be purified, for example using Actigel beads containing the covalently bound peptide.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten. polypeptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid. Of particular interest, are monoclonal antibodies which recognize the proline-rich domain of $p70\beta^{S6k}$.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as Fav, $_{sc}$FV, Fab, Fab', or $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin. Antibodies may preferably also be human, humanized or chimeric variants of the foregoing. Such antibodies can be less immunogenic when administered to a subject. Methods of producing humanized or chimeric antibodies are well known in the art. The antibodies contemplated also include different isotypes and isotype subclasses (*e.g.*, IgG_1, IgG_2, IgM, to name a few). These antibodies can be prepared by raising them in vertebrates, in hybridoma cell lines or other cell lines, or by recombinant means. For references on how to prepare these antibodies, see E. Harlow and D. Lane, ANTIBODIES: A LABORATORY MANUAL (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1988); Kohler and Milstein, (1976) E. J. Immunol. 6:511; Queen *et al.* U.S. Patent NO. 5,585,089; and Riechmann *et al.*, Nature 332:323 (1988).

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of receptor

can also be produced in the context of chimeras with multiple species origin.

In an alternative format, a specific activity of a protein of the invention may be assayed, such as the ability of the protein to phosphorylate a substrate such as polypeptides of the S6 protein. For example, $p70\beta^{S6k}$ has been demonstrated to phosphorylate the S6 protein and a synthetic peptide, RRLSSLRASTSKSESSQK (SEQ ID No. 8). The sequence comprising the synthetic peptide is located in the C-terminus of the S6 protein and is known to contain the five phosphorylation sites targeted by $p70\alpha^{S6k}$. Cell lines or populations are exposed under appropriate conditions to the agent to be tested. Agents which modulate the kinase activity of the protein of the invention are identified by assaying the kinase activity of the protein from the exposed cell line or population and a control, unexposed cell line or population, thereby identifying agents which modulate the kinase activity of the protein. Polypeptides of the S6 protein, such as the above examples, are useful positive controls in identifying additional $p70\beta^{S6k}$ substrates.

Kinase assays to measure the ability of the agent to modulate the kinase activity of a protein of the invention are widely available such as the assays disclosed by Mishima *et al.* (1996) J. Biochem. 119: 906-913) and Michnoff *et al.* (1986) J. Biol. Chem. 261: 8320-8326. Alternative assay formats include actin-myosin motility assays such as those disclosed by Kohama *et al.* (1996) TIPS 17: 284-287 or Warrick *et al.* (1987) Ann. Rev. Cell. Biol. 3: 379-421.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. As described

in the Examples, there are proposed binding sites for ATP/GTP and calmodulin as well as cAMP/cGMP kinase sites, TyrP sites and Ser/Thr kinase (catalytic) sites in the protein having SEQ ID No.2. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up these sites. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the ATP or calmodulin binding sites or domains.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of proteins of the invention. Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies.

J. Uses for Agents that Modulate at Least One Activity of the S6 Kinase Protein.

As provided in the Examples, the proteins and nucleic acids of the invention, such as the protein having the amino acid sequence of SEQ ID No.2, are involved in ribosomal function. Agents that modulate or down-regulate the expression of the protein or agents such as agonists or antagonists of at least one activity of the protein may be used to modulate biological and pathologic processes associated with the protein's function and activity.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by a protein of the invention. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological process mediated by a protein of the invention may include binding of substrates such as ATP, GTP or calmodulin or phosphorylation of a substrate, such as the S6 protein.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, expression or up-regulation of expression of a protein of the invention may be associated with certain diseases. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, a disease may be prevented or disease progression modulated by the administration of agents which reduce or modulate in some way the expression or at least one activity of a protein of the invention.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which modulate expression or at least one activity of a protein of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise about 0.1 to

100 μg/kg body weight. The preferred dosages comprise about 0.1 to 10 μg/kg body weight. The most preferred dosages comprise about 0.1 to 1 μg/kg body weight. In tissue culture, optimal dosage ranges for drugs such as wortmannin and rapamycin range from about 500 pM to about 1000 nM. Less optimum ranges include about 10 pM to about 10 mg.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be coadministered along with other compounds typically prescribed for these

conditions according to generally accepted medical practice. The compounds of this invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

EXAMPLE 1

Amino Acid Sequence of $p70\beta^{S6k}$ and comparison with $p70\alpha^{S6k}$

Materials and Methods. Restriction enzymes and DNA modification enzymes were obtained from standard commercial sources and used according to manufacturer's recommendations. Oligonucleotides which were used for sequencing of $p70\beta^{S6k}$ and various PCR fragments were synthesized by Genosys or Japan Bioservice, Inc. The pcDNA1 and pcDNA3 mammalian expression vectors were from Invitrogen. The pGEX-4T vector, glutathione-Sepharose-4B and HiTrapQ columns were purchased from Pharmacia. cDNA of rat p70α-I was a gift from Dr. Joseph Avruch (Diabetes Unit, Massachusetts General Hospital). Rapamycin and PDGF BB were purchased form Calbiochem. Wortmannin was purchased from Sigma.

Cell cultures and Antibodies. A porcine aortic endothelial cell line (PAE-PDGF-R), stably expressing the human PDGF-β receptor, was maintained in HAM's F12 medium containing 10% fetal calf serum (FCS). CHO cells stably overexpressing human insulin receptors (CHO-IR cells) and HEK293 cells were maintained and cultured as described earlier (Hara *et al.*, 1998 J. Biol. Chem. 273: 14484-14494) in HAM's F12 medium or Dulbecco's modified Eagles minimal essential medium (DMEM) supplemented with 10% FCS, respectively. Anti-FLAG monoclonal M2

antibody was purchased from Eastman Kodak Corp. Anti-phosphopeptide antibodies against proline-directed site Ser434 of p70α-I were purchased from New England Biolabs. Polyclonal antibodies raised against the C-terminal 104 amino acids fragment of $p70\alpha^{S6k}$ were from Dr. Joseph Avruch. A GST fusion protein containing amino acids 443-495 of $p70\beta^{S6k}$ (p70βC Ab) was used to raise polyclonal antibodies specific for $p70\beta^{S6k}$. Immunoreactive sera were affinity-purified on an Affigel matrix containing the GST/$p70\beta^{S6k}$-terminal fusion protein.

Fractionation of cell extracts. HEK293 cells were starved in DMEM medium for 16 h and then treated with 15% FCS for 10 min or 200 nM rapamycin for 30 min. After treatment, cells were lysed in ice-cold buffer A (20mM Tris/HCl pH 7.5, 20 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM β-glycerophosphate, 1 mM-DTT, 1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin) and the lysates centrifuged at 4°C for 20 min at 10,000xg. Supernatants were filtered through a 0.45 μM filter and then loaded onto a HighTrapQ Sepharose column (1.0 ml column volume) equilibrated in Buffer A. The column was washed extensively in buffer A and bound proteins eluted with a linear gradient of NaCl (20 - 500 mM). Aliquots of eluted proteins were subjected to SDS- PAGE and immunoblotted with anti-phosphopeptide antibodies directed against proline-directed site Ser-434 of $p70\alpha^{S6k}$ or anti-peptide antibodies against the carboxyl terminal end of $p70\alpha^{S6k}$.

Construction and screening of a HEK293 Uni-ZAP library and DNA sequencing analysis. Total RNA was isolated from HEK293 cells as described (Hara *et al.*, 1998; Chomczynski *et al.*, 1987 Analytical Biochem. 162: 156-159), and poly(A)+ mRNA was selected by using Dynabeads mRNA purification kit (Dynal). An oligo(dT)-primed library was constructed in UNI-ZAP XR vector from 5 mg of HEK293 mRNA, using the Uni-ZAP cDNA synthesis kit (Stratagene). Packaging into phages was carried out by using Gigapack III Gold Packaging extracts (Stratagene). The cDNA encoding full length $p70\beta^{S6k}$ kinase was isolated by screening $1x10^6$ primary phages from HEK293 Uni-ZAP library with a ^{32}P-labeled 0.65 Kb *EcoRI/NotI* fragment derived from the EST clone GenBank Accession No. AA410355 (Hillier *et al.*,

published on GenBank, 1997). Positive clones were confirmed, isolated in second-round screening and rescued as Bluescript plasmids by *in vivo* excision (Stratagene). PCR amplification and restriction mapping were used for primary characterization of isolated clones. Sequencing analysis of selected clones was performed on an Applied Biosystem 373A DNA automatic sequencer (PE Applied Biosystems).

Results. The p70α^{S6k} is activated by multiple phosphorylation within the pseudosubstrate and catalytic domains in response to extracellular stimuli, including serum, growth factors and hormones. Phosphospecific antibodies directed against phosphorylated sites of p70α^{S6k} have been recently developed: Phospho-p70α^{S6k} (Ser434) and Phospho-p70α^{S6k} (Thr444/Ser447). Both antibodies were shown to recognize specifically phosphorylated forms of p70α^{S6k}, and this recognition was found to be sensitive to rapamycin. To compare the phosphorylation state and chromatographic behavior of p70α^{S6k} from cells stimulated with serum and treated with or without rapamycin, HEK293 cells were starved in DMEM medium for 16 h and then stimulated with 15% serum for 10 min prior treatment with or without 200 nM of rapamycin for 30 min. Cell extracts were fractionated using HighTrapQ Sepharose columns. Proteins were resolved on SDS-PAGE and immunoblotted with anti-phosphopeptide antibodies Ser434 and S444/T447 or antibodies specific for p70α^{S6k}. In total lysates of serum stimulated HEK293 cells, this antibody recognizes specifically phosphorylated versions of p70α^{S6k} isoforms (p70 and p85). However, when cell lysates were fractionated and separated on SDS-PAGE, several additional bands appeared on the immunoblot together with the p70 and p85 isoforms of p70α^{S6k}, including p190, p110, p90 and p60 (not shown). It is important to note that the recognition of these proteins by phosphospecific S434 antibodies was sensitive to rapamycin, indicating the specificity for the phosphorylated epitope. As expected, the anti-peptide antibodies specific for the p70α^{S6k} recognized 85-kDa and 70-kDa bands, which correspond to p70α-I and p70α-II, respectively. S434 is located in the autoinhibitory region of the p70α^{S6k}, which is highly conserved among different species of p70α^{S6k}, including Drosophila (Stewart *et al.*, 1996 Proc. Natl Acad. Sci. USA 93:

10791-10796; Watson *et al.*, 1996 Proc. Natl Acad. Sci. USA 93: 13694-13698). As phosphorylation of the S434 site is sensitive to rapamycin, it is possible to speculate that p190, p110, p90 and/or p60 may represent novel rapamycin-sensitive $p70\alpha^{S6k}$-related kinases.

Molecular cloning of novel $p70\alpha^{S6k}$. Peptide sequences which were used for raising anti-phosphopeptide antibodies S434 of p70α-I were taken to search the expressed sequence tag (EST) databases. This search generated hundreds of EST clones that showed high degree of homology to the query sequence. Extensive analysis of these clones allowed us to isolate several clones which were highly homologous to the 434 peptide, but did not match to cDNA clones from EMBL or Swissprot databases. Further characterization of these sequences indicated that two nearly identical clones (GenBank Accession Nos. AA284234 and AA410355) exhibited strong homology to the kinase extension domain of the protein kinase A (PKA) family of serine/threonine (S/T) kinases. Additionally, the homology in the kinase extension domain extended into the putative autoinhibitory domain, which is unique for the $p70\alpha^{S6k}$ (less than 75% identity between $p70\alpha^{S6k}$ and the unknown potential S6 kinase). However, the homology with $p70\alpha^{S6k}$ dropped significantly downstream of the kinase extension and autoinhibitory domains, suggesting that these clones encoded a novel kinase of this family. Based on these data, we decided to further characterize these clones. Both EST clones were obtained from the UK HGMP Resource Center. Restriction mapping indicated that the GenBank Accession Nos. AA284234 and AA410355 clones contain short inserts of 0.6 kB and 0.65 kB respectively. Sequence analysis showed that these clones are identical to each other in an overlapping region and may encode a partial open reading frame (ORF), which shows very strong homology to the kinase extension and autoinhibitory domains of the $p70\alpha^{S6k}$. These ESTs did not contain a full gene nor was there a protein coding sequence previously identified in these ESTs. Furthermore, four of the five proline-directed Ser/Thr phosphorylation sites located in an autoinhibitory pseudo-substrate domain of $p70\alpha^{S6k}$ were conserved in the clones of $p70\beta^{S6k}$. Immediately after the autoinhibitory pseudo-substrate domain, the homology

between p70α^{S6k} and p70β^{S6k} was very low (12% identity). Taking in account these findings, we proceeded to clone the full length cDNA clone encoding this potentially novel kinase.

Since several rapamycin-sensitive bands were found in the HEK293 cells with the use of anti-Ser434 phosphospecific antibodies, a library from this cell line was created. We screened 10^6 primary clones from the Uni-Zap/HEK293 library with a full length insert from EST clone AA410355 and isolated 12 positive clones. Sequence analysis of rescued plasmids allowed us to identify one clone, which contained an open reading frame of 495 amino acids (Figure 2A). The C-terminus of this clone was found to be identical to the sequence of the EST clone AA410355, which was used for screening.

By analogy to p70α^{S6k}, the novel cDNA, encoding p70β^{S6k}, could potentially encode two isoforms as a result of alternative start codons. If this is the case, the shorter isoform may utilize an ATG codon which is 13 amino acids (aa) downstream of the first methionine and may encode a protein of 482 amino acids. Two potential isoforms were termed p70β-I (495 aa long) and p70β-II (482 aa long). The presence of additional 13 aa at the N-terminus of p70β-I isoform may determine its subcellular localization in the nucleus due to the presence of a putative nuclear localization sequence (RGRRARG, amino acid numbers 3-9 of SEQ ID No. 2). The overall structure of p70β^{S6k} is similar to that of p70α^{S6k}. p70α^{S6k} and p70β^{S6k} share 70% identity and 85% similarity on protein level. The p70β^{S6k} kinase consists of the amino-terminal non-catalytic region, a catalytic domain, a kinase extension and a carboxyl-terminal non-catalytic tail, whose amino acid identity corresponds to domains of p70α^{S6k} is 40%, 83%, 80%, and 47%, respectively (Figure 2B). The strong argument that this clone encodes a novel p70 S6 kinase is the presence of the autoinhibitory pseudosubstrate domain, which is not present in any other known kinases.

p70α^{S6k} undergoes a multi-site phosphorylation in response to stimulation by insulin or mitogens. Such multiple phosphorylation sites are also well conserved in p70β^{S6k} (Figure 2B). It also contains 3 sets of phosphorylation sites, similar to p70α^{S6k}:

(i) a set of Ser/Thr-Pro motifs clustered in an autoinhibitory pseudosubstrate domain (Ser423, Ser430, Ser436, Ser441 in $p70\beta^{S6k}$ correspond to Ser 434, 441, and 447, Ser 452 in $p70\alpha^{S6k}$; (ii) a second set includes Ser383 and Thr401 which is located in the kinase extension domain and corresponds to Ser394 and Thr412 in $p70\alpha^{S6k}$; (iii) a third set consists of Thr251 which resides in the activation T-loop of kinase domain and corresponds to Thr252 in $p70\alpha^{S6k}$. The greatest difference between the $p70\alpha^{S6k}$ and the $p70\beta^{S6k}$ sequences are in the amino-terminal non-catalytic region (40% identity and 60% similarity) and the carboxyl-terminal non-catalytic tail (47% identity and 66% similarity). $p70\beta^{S6k}$ also contains proline-rich sequences at the C-terminus, that may mediate the interaction with SH3-domain-containing molecules.

EXAMPLE 2

Tissue Specific Expression of $p70\beta^{S6k}$

Materials and Methods. Northern blot analysis was performed using commercial nylon membranes pre-bound with 2 μg of gel-separated poly(A)+ RNA samples obtained from various human tissues or tumor cell lines from Clontech. The following probes were used for the detection of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ messages: (i) a 476-base pair (bp) *HindIII* fragment spanning 56 bp of 3' end coding region and 420 bp of 3' non-coding region of the human $p70\alpha^{S6k}$ (EST clone, AA425599), (ii) a 650-bp fragment spanning 518 bp upstream of the stop codon and about 130 bp of non-coding region of the human $p70\beta^{S6k}$ (EST clone, AA410355). Human β-actin cDNA probe was used as a negative control (Clontech). These probes were labeled by Multiprime DNA labeling system (Amersham) and separated from unincorporated [γ^{32}P]dCTP by Nuctrap push columns (Stratagene). Northern blots were pre-hybridized with ExpressHyb solution and hybridized with labeled probes according to manufacturer's recommendations. After extensive washing with 2X SSC, 0.05% sodium dodecyl sulfate (SDS) at room temperature, and twice with 0.1X SSC, 0.1% SDS at 50°C, the localization of bound probes on membranes was identified by autoradiography or with the use of the PhosphoImager.

Results. In order to compare expression patterns of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ in human tissues and cell lines, 3' prime coding and non-coding regions, which exhibit low level of homology between both the α and β S6 kinases, were used as probes. Northern blot analysis using poly(A)+ RNA isolated from human tissues revealed a single 2.2 kb transcript for $p70\beta^{S6k}$, while $p70\alpha^{S6k}$ probe specifically hybridized to 3.4 kb and 7.4 kb transcripts (Figure 3A). The expression pattern of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ transcripts is remarkably similar, showing ubiquitous expression in all tissues. Highest expression levels were found in spleen, skeletal muscle and peripheral blood leukocytes, whereas brain, lung and kidney showed the lowest expression of transcripts for both S6 kinases. The only significant difference on the level of mRNA expression between $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ was found in liver. The expression of $p70\beta^{S6k}$ mRNA in liver is 2-3 times higher than that of $p70\alpha^{S6k}$. Liver possesses a high concentration of $p70\alpha^{S6k}$ and was originally used by several groups to purify $p70\alpha^{S6k}$ for biochemical studies and protein sequencing analysis (Banerjee *et al.*, 1990 Proc. Natl Acad. Sci. USA 87: 8550-8554; Kozma *et al.*, 1990 Proc. Natl Acad. Sci. USA 87: 7365-7369).

We also analyzed the expression of $p70\beta^{S6k}$ mRNA in tumor cell lines using the same probe as for the analysis of tissue distribution. A single transcript of the same size as in human tissues, 2.2 kb, was found to be highly expressed in HeLa and K562 cells, but was barely detectable in HL-60, MOLT-4 and melanoma G361 cell lines (Figure 3B).

EXAMPLE 3

Phosphorylation of the ribosomal protein S6 and its C-terminal synthetic peptide by $p70\alpha^{S6k}$ and $p70\beta^{S6k}$

Materials and Methods. Expression of GST/$p70\beta^{S6k}$ fusion protein in bacteria. A PCR-based strategy was used to make a bacterial expression plasmid for GST/$p70\beta^{S6k}$ fusion protein. A cDNA fragment encoding 443-495 amino acids of $p70\beta^{S6k}$ was amplified by PCR and cloned into the pGEX-4T expression vector (Pharmacia). This construct was transformed into *E. coli* XL1-Blue competent cells

(Stratagene) and the expression of the GST/p70β^{S6k} fusion protein was induced by isopropyl β-D-thiogalactopyranoside (IPTG). The GST/p70β^{S6k} fusion protein was purified by using glutathione-Sepharose-4B beads according to manufacturer's recommendation (Pharmacia). After SDS-PAGE analysis, affinity purified fusion proteins were dialyzed against 20 mM (Tris pH 7.4), 150 mM NaCl, 50% Glycerol and stored at -20°C. This preparation of the GST/p70β^{S6k} C-terminal fragment was used for the production of polyclonal antibodies specific for p70β^{S6k}.

Construction of mammalian expression plasmids. The full length coding sequence, corresponding to the p70β-I (I-495 amino acids) was amplified by PCR using human cDNA clone N53 isolated from HEK293 library as a template and a panel of specific oligonucleotides. Amplified constructs were digested with appropriate enzymes, gel purified and cloned into the pcDNA1 vector in-frame with N-terminal FLAG epitope.

Amino-terminal EE-tagged p70α-II and p70β-II constructs were created by a PCR-based cloning strategy. This was achieved by using specific oligonucleotides containing EE-tag sequence and appropriate restriction sites. The cDNA encoding full length human p70β^{S6k} (clone 53) and rat p70α^{S6k} were used as templates. The resulting PCR fragments were digested with restriction enzymes, gel purified and cloned into the pcDNA3 expression vector. The construction of the pMT2 FLAG p70α-I was described previously (Hara *et al.*, 1998). All constructs generated by a PCR-based approach were verified by sequencing. A Qiagen plasmid Midi kit was used to purify plasmid DNAs for transient transfections. The introduction of the FLAG-tag and the EE-tag sequences at the N-terminus of p70α^{S6k} and p70β^{S6k} allows the study of recombinant proteins via the use of specific antibodies.

Results. To test if the isolated p70β^{S6k} cDNA would encode a functional kinase capable of phosphorylating ribosomal protein S6, a cDNA fragment encoding a short version of p70β^{S6k} (p70β-II) was subcloned into mammalian expression vector in frame with EE-tag or Flag-tag epitopes. These constructs were transfected into HEK293 cells using lipofectAMINE under conditions recommended by the manufacturer. The

expression of recombinant p70β^{S6k} was analyzed by immunoprecipatation or western blotting with EE-tag or Flag-tag antibodies. Both constructs express the protein of approximately 60kDa. The expression level of p70β^{S6k} was comparable to that of p70α-I and p70α-II isoforms, when expressed in HEK293 cells.

Anti-p70β^{S6k} polyclonal antibodies were generated using synthetic peptides corresponding to the carboxy terminal 15 amino acids of p70β^{S6k}. These peptides were coupled to KLH and then injected into rabbits using standard procedures. Immune sera was purified using Affigel beads containing covalently cross-linked carboxy terminal peptide.

To determine whether the putative p70β^{S6k} was indeed a novel ribosomal protein S6 kinase, the recombinant p70β^{S6k} was expressed in HEK293 cells, immunoprecipitated with anti-EE-tag antibodies and an *in vitro* kinase reaction performed in the presence of purified 40S ribosomal subunit. As shown in Figure 4A, p70β^{S6k} (p70β-II isoform) phosphorylates the S6 protein *in vitro* nearly as efficiently as p70α^{S6k}. We also tested the ability of p70β^{S6k} (p70β-II isoform) to phosphorylate a synthetic peptide representing C-terminus of S6 protein, which contains all sites known to be phosphorylated by p70α^{S6k}. Figure 4B demonstrates that p70β^{S6k} (p70β-II isoform) also phosphorylates this peptide but with slightly lower efficiency than observed with p70α^{S6k}. Therefore, p70β^{S6k} (p70β-II isoform) may not phosphorylate the same sites of the ribosomal protein S6 as does p70α^{S6k}.

The intrinsic activity of the p70β^{S6k} is significantly lower than p70α^{S6k}. The absence of one phosphorylation site in the autoinhibitory domain of p70β^{S6k} may be responsible for this decrease in intrinsic activity.

EXAMPLE 4

Stimulation of p70β^{S6k} Activity by insulin, serum and TPA

Materials and Methods. HEK293 cells or CHO-IR cells were transfected with plasmids containing p70α^{S6k} and p70β^{S6k} sequences using lipofectAMINE under conditions recommended by the manufacturer (Gibco-BRL). Two days later,

transfected cells were frozen in liquid nitrogen and stored until lysis. After cell extraction, the lysates were subjected to immunoprecipitation and/or immunoblot analysis. If cells were to be stimulated, they were starved in medium without FCS for 16 h and then stimulated with 10^{-7} M insulin for 10 min, 15% FCS for 10 min, 500 nM TPA for 30 min or vehicle alone. PAE-PDGF-R cells were transfected with appropriate plasmids using lipofectAMINE under conditions recommended by the manufacturer. After 24 h transfected cells were serum-starved for 16 h and stimulated with 20 ng/ml PDGF BB (Calbiochem) for 20 min. Control cells were treated with the vehicle under the same conditions.

Results. The effect of various extracellular stimuli on the $p70\beta^{S6k}$ activity was studied in different cell lines, transiently transfected with Flag-tagged or EE-tagged versions of $p70\beta^{S6k}$. The activation of $p70\beta^{S6k}$ (p70β-II isoform) by insulin was analyzed in CHO-IR cells, which stably over expresses the insulin receptor. As shown in Figure 5A, treatment of CHO-IR cells with insulin induces $p70\beta^{S6k}$ (p70β-II isoform) activity towards ribosomal S6 protein by 2.8 fold. In the same cell line and under the same conditions, the activity of $p70\alpha^{S6k}$ was activated 3.5 fold with insulin treatment (Figure 5A). Almost equal amounts of $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ (p70β-II isoform) were expressed in cells and used in an *in vitro* kinase assay after immunoprecipitation. In addition, both serum and TPA also stimulated $p70\beta^{S6k}$ (p70β-II isoform) phosphorylation of the ribosomal protein S6 (Figure 5A).

Using PAE-PDGF-R cells, the activation of the $p70\alpha^{S6k}$ and $p70\beta^{S6k}$ (p70β-II isoform) by PDGF was examined. This cell line stably over expresses the PDGF receptor, and the activation of $p70\alpha^{S6k}$ in response to PDGF was shown to be very efficient (Figure 5B). We found that PDGF stimulation of these cells leads to a rapid activation of recombinant $p70\beta^{S6k}$ (p70β-II isoform).

These data indicate that $p70\beta^{S6k}$ is activated by a number of extracellular stimuli in a very similar way as $p70\alpha^{S6k}$. However, activation in the PAE-PDGF-R cells was 30 fold for $p70\beta^{S6k}$ and only 3.4 fold for $p70\alpha^{S6k}$.

EXAMPLE 5

Effects of rapamycin and wortmannin on p70β^{S6k} and p70α^{S6k}

Materials and Methods. Treatment of transfected cells with rapamycin or wortmannin was performed as follows: 48 h after transfection, the cells were treated with various concentrations of rapamycin or wortmannin for 30 min.

Results. These data in Examples 4 and 5 indicate that p70β^{S6k} is activated by a number of extracellular stimuli in a similar fashion to p70α^{S6k}. The two fungal inhibitors, wortmannin and rapamycin, specifically inhibit activation of p70α^{S6k} via PI3-kinase- and mTOR-dependent pathways respectively. Thus, the effects of both inhibitors on p70β^{S6k} activity were examined. After p70α-I and p70β-II were transiently expressed in HEK293 cells, cells were maintained in DMEM containing 10% FCS and then treated with various concentrations of rapamycin or wortmannin. We found that the activity of p70α^{S6k} and p70β^{S6k} were inhibited by rapamycin and wortmannin in a dose dependent manner (Figures 6A and B, upper panel). However, it appears that p70β^{S6k} is less sensitive to rapamycin and wortmannin, when compared with p70α^{S6k}. This difference is more obvious at lower concentration of inhibitors. In the presence of 20 nM rapamycin the inhibition of the p70α^{S6k} is 92%, while only 46% for p70β^{S6k}. Addition of 100 nM of wortmannin inhibits 86% of p70α^{S6k} activity and 62% of p70β^{S6k} activity. The inhibition of p70β^{S6k} by rapamycin and wortmannin is lower than that observed for p70α^{S6k} indicating different mechanisms of regulation exist for p70β^{S6k}.

EXAMPLE 6

Interaction of the p70β^{S6k} with different GST/SH3 fusion proteins

Materials and Methods. The EE-tag/p70β^{S6k} was transiently over expressed in HEK293 cells as described above. Transfected cells were lysed in buffer A (50 mM-Tris/HCl pH = 8.0, 1% NP-40, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 20 mM sodium β-glycerophosphate, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 2 μg/ml aprotinin) two days after transfection. After

centrifugation at 12,000 rpm for 20 min, the supernatants were incubated for 2 hrs with Protein A beads pre-coupled with anti-EE antibodies. Beads were washed with lysis buffer before different GST/SH3 fusion proteins (1.5 mg each) were added to separate immunoprecipitation reactions. Two hours later, beads were washed extensively in lysis buffer, bound proteins separated on the SDS-PAGE and transferred to the PVDF membrane. Specific interaction of the GST/SH3 domains with pre-bound EE-tag/p70β^{S6k} was assessed by immunoblotting with the anti-GST antibodies.

Results. The C-terminus of the p70β^{S6k} contains proline-rich sequences which are not present in p70α^{S6k}. Src-homology region 3 domains (SH3 domain) are present in many signaling and cytoskeletal molecules and interact specifically with proline-rich sequences which form left-handed helixes. Sequence analysis of the proline-rich region in p70β^{S6k} indicates the presence of several putative SH3 domain binding motifs. Therefore, the ability of p70β^{S6k} (p70β-II isoform) to interact with a panel of SH3 domains was examined. In this experiment, the EE-tag/p70β^{S6k} was transiently over expressed in HEK293 cells and immunoprecipitated with anti-EE antibodies coupled to Protein G Sepharose. The resulting immunoprecipitates were incubated with different GST/SH3 domain fusion proteins. After extensive washing, specific interaction between p70β^{S6k} and SH3 domains was analyzed by SDS-PAGE and immunoblotting with anti-GST antibodies. As shown in Figure 7, several SH3 domains, including those of GAP, Src, Fgr exhibited specific interaction towards p70β^{S6k}.

EXAMPLE 7

Immunoprecipitation and Western blot analysis of transiently expressed p70β-I and p70β-II

Materials and Methods. Anti-p70β^{S6k} polyclonal antibodies were generated using a synthetic peptide corresponding to the C-terminal tail of p70β^{S6k}. The peptide was coupled to KLH, and rabbits were immunized using standard procedures. Immune sera harvested obtained from the immunized rabbits was purified using affinity chromatography on Affigel beads containing covalently cross-linked C-terminal

peptides of p70β^{S6k}.

HEK293 cells were transfected with pcDNA1 along, pcDNA1/Flag-p70α-I, pcDNA1/Flag-p70β-I, or pcDNA1/Flag-p70β-II. Two days after transfection with one of these plasmids, cell lysates were prepared. Proteins were immunoprecipitated using the p70β^{S6k} C-terminal affinity purified polyclonal antibodies or anti-Flag monoclonal antibodies. Immunoprecipitates were resolved on SDS PAGE and proteins transferred to PVDF membranes. The PVDF membranes were immunoblotted using anti-Flag monoclonal antibodies or p70β^{S6k} C-terminal antibodies as indicated in Figure 8.

Results. The expression of both p70β^{S6k} isoforms was analyzed in HEK293 cells using anti-p70β^{S6k} C-terminus specific polyclonal antibodies. Both p70β^{S6k} isoforms were found to be specifically immunoprecipitated with anti-p70β^{S6k} antibodies, but not p70α-I, as confirmed by anti-Flag and anti-p70β^{S6k} immunoblotting. It was found that the p70β-I isoform which encodes a 495 amino acid protein, is translated into a protein which migrates in a SDS-PAGE gel at approximately 70 kD. The p70β-II isoform, which is a truncated form of p70β-I lacking 13 amino acids at the amino terminus of p70β-II, migrates in a SDS-PAGE gel at approximately 60 kD.

EXAMPLE 8

Generation of activated variants of p70β^{S6k} (T401D) and p70α^{S6k} (T412D).

Materials and Method. Activated variants of p70β^{S6k} and p70α^{S6k} were made by site-directed mutagenesis. Oligonucleotide primers, specific to the site to be mutated and complimentary to opposite strands of p70β^{S6k} and p70α^{S6k} sequences were generated as recommended by manufacturer (Stratagene). Site-directed mutagenesis was carried out using pcDNA3/ Glu-tag-p70β^{S6k} and pcDNA3/Glu-tag- p70α^{S6k} expression vector/plasmids as templates, according to the recommended protocol (Stratagene). All mutations were verified by DNA sequencing. Expression of mutated forms of both kinases was analyzed by immunobloting with anti-Glu-tag antibodies. The activity of normal and mutated forms of both kinases was measured by in vitro S6 kinase assay. 40S ribosomal subunit was used as a substrate in this reaction.

Results. Studies on $p70\alpha^{S6k}$ demonstrate that this kinase is activated by multiple phosphorylations in response to growth factors or hormones (Fig. 9). A number of kinases that can phosphorylate $p70\alpha^{S6k}$ *in vitro* and *in vivo* have been identified, including cdc2, MAPK, SAPK, p38, TOR and PDK1. However, very little is known about the process of dephosphorylation, which is essential for the inactivation of the kinase.

We have generated an activated form of $p70\beta^{S6k}$ by substituting putative phosphorylation site Thr 401 with Aspartic acid (Asp) ("$p70\beta^{S6k}$ (T402D)") as shown in Fig 10. Transfection studies in HEK293 cells and S6 kinase assays indicated that $p70\beta^{S6k}$ (T401D) mutant is in an activated state in unstimulated cells, when compared with wild type kinase (3 times higher activity, as shown in Fig 11). We have also created an activated version of $p70\alpha^{S6k}$ (T412D), which showed a greater state of activation (18 fold activation, Fig. 12).

It will be apparent to the skilled artisan that activated variants of both kinases such as disclosed above can be used in the search for binding partners. Binding partners or molecules, such as phosphatases, are considered to form tighter and more stable complexes with such irreversibly activated kinases.

EXAMPLE 9

Identification of $p70\beta^{S6k}$-binding partners.

Materials and Methods. HEK293 cells are transfected using lipofectAMINE (as recommended by manufacturer, Gibco-BRL) with pcDNA3 expression vectors encoding activated variants of $p70\beta^{S6k}$ (T401D) and $p70\alpha^{S6k}$ (T412D). Binding partners that preferentially associate with $p70\beta^{S6k}$ may be identified by comparing the profile of proteins precipitated from activated $p70\beta^{S6k}$ expressing cells to the profile of activated $p70\alpha^{S6k}$ expressing cells and/or negative control cells. Cells transfected with the pcDNA3 plasmid alone, may be used as a negative control in this experiment. Two days after transfection, cells are lysed in extraction buffer: 50 mM Tris/HCl (pH8.0); 120 mM NaCl; 20 mM NaF; 20 mM b-glycerophosphate; 1 mM EDTA, (pH 8.0); 6

mM EGTA; 1% NP-40; 1 mM DTT. The following protease and phosphatase inhibitors are added to the extraction buffer just before cell lysis: 5 mM Benzamidine; 1 mM PMSF; 1mg/ml of aprotinin; 0.125 mM NaVO4; Pepstatin; and Leupeptin.

The resulting cell lysate is centrifuged at 14,000 rpm for 20 min at 4°C to remove the insoluble fraction. If the lysate is not used immediately it is stored at -80°C until needed. The protein concentration of the samples is measured using a Coomassie Protein Assay reagent (Pierce) at 595 nm. An equal amount of supernatant from each sample is added to fresh 1.5 ml tubes and the volumes is equalized using lysis buffer. Affinity purified anti-Glu antibody is added to the supernatant and incubated on the wheel for 1 hr at 4°C. Protein-G sepharose beads, pre-washed in lysis buffer, are used to bring down immune complexes.

After extensive washing in lysis buffer (4x), 2x sample buffer is added to the beads. Bound proteins are eluted from the beads by boiling and separated by SDS-PAGE electrophoresis. Separated proteins are silver stained and the pattern of associated proteins is analyzed.

Results. The pattern of associated proteins are compared between activated variants of $p70\beta^{S6k}$ and $p70\alpha^{S6k}$ kinases. Mutated variants of both kinases are transiently expressed in HEK293 cells as Glu-tag fusion proteins. The presence of a Glu-tag epitope at the N-terminus of $p70\beta^{S6k}$ and $p70\alpha^{S6k}$ allows specific immunoprecipitation of activated kinases from transfected cells. The skilled practioner will recognize that the Glu-tag fusion is not necessary to the invention and that similar results could be obtained with antibodies specific to each or both of the activated variants in the absence of a fusion epitope.

The skilled practioner will recognize that binding partners or polypeptides that preferentially bind to activated $P70\beta^{S6K}$ can be isolated by one or more standard techniques such as immunoprecipitation, hplc, fplc, column chromatography or preparative electrophoresis.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without

departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

WHAT IS CLAIMED:

1. An isolated nucleic acid molecule selected from the group consisting of a nucleic acid molecule comprising the sequence of SEQ ID No.1 (the nucleotide sequence of the $p70\beta^{S6k}$); and a nucleic acid molecule which encodes a $p70\beta^{S6k}$ and which hybridizes to a nucleic acid molecule having the sequence of SEQ ID No.1 under stringent conditions.

2. An isolated nucleic acid molecule which encodes a protein having SEQ ID No.2 (the $p70\beta^{S6k}$) or a protein having one or more conservative amino acid substitutions in SEQ ID No.2.

3. An isolated nucleic acid molecule selected from the group consisting of a nucleic acid molecule comprising the sequence of SEQ ID No.1 (the nucleotide sequence of the $p70\beta^{S6k}$); a nucleic acid molecule which encodes a $p70\beta^{S6k}$ and which hybridizes to a nucleic acid molecule having the sequence of SEQ ID No.1 under stringent conditions, and a nucleic acid molecule at least about 65% sequence identity with SEQ ID No.1.

4. An isolated nucleic acid molecule which encodes a protein having SEQ ID No.2 (the $p70\beta^{S6k}$), a protein with at least 75% identity to SEQ ID No.2, or a polypeptide fragment of SEQ ID No.2.

5. An isolated polypeptide which phosphorylates a ribosomal S6 protein and is encoded by a nucleic acid molecule of claim 1 or claim 2.

6. A method of identifying an agent which modulates $p70\beta^{S6k}$ mediated phosphorylation of a ribosomal S6 subunit comprising the steps of:

exposing $p70\beta^{S6k}$ and a ribosomal S6 subunit to the agent; and

determining whether the agent modulates $p70\beta^{S6k}$ mediated phosphorylation of the ribosomal S6 subunit.

7. A method of modulating protein synthesis or cellular proliferation comprising the step of administering an agent which modulates $p70\beta^{S6k}$ phosphorylation of a ribosomal S6 subunit.

8. A method of identifying an agent that modulates a kinase or a phosphatase induced regulation of $p70\beta^{S6k}$ activity comprising the steps of:

exposing $p70\beta^{S6k}$ and the kinase which phosphorylates $p70\beta^{S6k}$ to an agent; and

determining whether the agent modulates the kinase or the phosphatase induced regulation of $p70\beta^{S6k}$ activity.

9. The method of claim 8, wherein the kinase which phosphorylates $p70\beta^{S6k}$ is PKC.

10. A method of modulating protein synthesis or cellular proliferation comprising the step of administering an agent which modulates the phosphorylation of $p70\beta^{S6k}$.

11. A method of modulating cell cycle comprising the step of administering an agent which regulates the ability $p70\beta^{S6k}$ to bind with a ligand.

12. An antibody or antibody fragment which specifically binds to an epitope of $p70\beta^{S6k}$.

13. The antibody of claim 11, wherein the antibody is selected from the group consisting of a monoclonal antibody, human antibody, chimeric antibody, and humanized antibody.

14. An antibody of claim 11 wherein the epitope is a proline rich epitope of a $p70\beta^{S6k}$ protein.

15. A fusion protein comprising SEQ ID No.2 or a polypeptide fragment thereof fused to a heterologous protein.

16. A cell transformed with a nucleic acid molecule of any of claims 1-3.

17. A method of identifying a substrate of $p70\beta^{S6k}$ comprising the steps of:
exposing $p70\beta^{S6k}$ or a polypeptide fragment thereof to an agent; and
determining whether $p70\beta^{S6k}$ binds to the agent.

18. A method of identifying a substrate of $p70\beta^{S6k}$ comprising the steps of:
forming a mixture comprising $p70\beta^{S6k}$ and a candidate agent;
incubating said mixture under conditions conducive to phosphorylation by $p70\beta^{S6k}$; and
determining whether the candidate agent is phosphorylated.

19. A method of indentifying binding partners of $p70\beta^{S6K}$ comprising the step of incubating a first cellular extract with $p70\beta^{S6K}$, activated variants of $p70\beta^{S6K}$ or a fusion protein of claim 15.

20. The method of claim 19 further comprising incubating a second cellular extract with $p70\alpha^{S6K}$, activated variants of $p70\alpha^{S6K}$ or a fusion protein of $p70\alpha^{S6K}$ and comparing the first and second cellular extracts.

21. A method of indentifying binding partners of $p70\beta^{S6K}$ comprising the step of isolating a first a first cellular extract from a cell containing $p70\beta^{S6K}$, activated variants of $p70\beta^{S6K}$ or a fusion protein of claim 15.

22. The method of claim 19 further comprising isolating a second cellular extract from a cell containing $p70\alpha^{S6K}$, activated variants of $p70\alpha^{S6K}$ or a fusion protein of $p70\alpha^{S6K}$ and comparing the first and second cellular extracts.

23. An isolated polypeptide comprising an activated $p70\beta^{S6k}$.

24. The isolated polypeptide of claim 23 further comprising a mutation of Threonine 401 to Aspartic acid.

25. An isolated polypeptide that preferentially binds to an activated $p70\beta^{S6K}$ of claim 23.

26. The isolated polypeptide of claim 25 that preferentially binds to an activated $p70\beta^{S6k}$ of claim 24.

27. An antibody or antibody fragment that specifically binds to the isolated polypeptide of claims 25 or 26.

28. A method of determining whether a cell expresses aberrant cellular levels of $p70\beta^{S6k}$ comprising:
(a) determining the level of $p70\beta^{S6k}$ in a normal cell type;
(b) determining the level of $p70\beta^{S6k}$ in a test cell;
(c) comparing the level of $p70\beta^{S6k}$ in the normal cell to the $p70\beta^{S6k}$ level in the test cell.

29. The method of claim 28 wherein the level of $p70\beta^{S6k}$ is determined by finding the level $p70\beta^{S6k}$ RNA in a cell.

30. The method of claim 28, wherein the level of $p70\beta^{S6k}$ is determined by finding the level of $p70\beta^{S6k}$ protein in a cell.

31. A method of determining whether a cell expresses aberrant cellular levels of a $p70\beta^{S6k}$ binding partner comprising:
(a) determining the level of said binding partner in a normal cell;
(b) determining the level of said binding partner in a test cell;
(c) comparing the level of said binding partner in the normal cell to the binding partner level in the test cell.

32. A vector comprising the isolated nucleic acid of claim 2, operably linked to a promotor or transcription.

33. The vector of claim 32, further comprising one or more enhancers or upstream activating sequences.

34. The vector of claim 32, wherein the vector comprises pcDNA3.

35. A vector which encodes an activated $p70\beta^{S6k}$ of claims 23 or 24.

36. A DNA vector comprising a nucleic acid encoding a $p70\beta^{S6k}$ or an activated $p70\beta^{S6k}$ fusion protein.

FIG. 1A

FIG. 1B

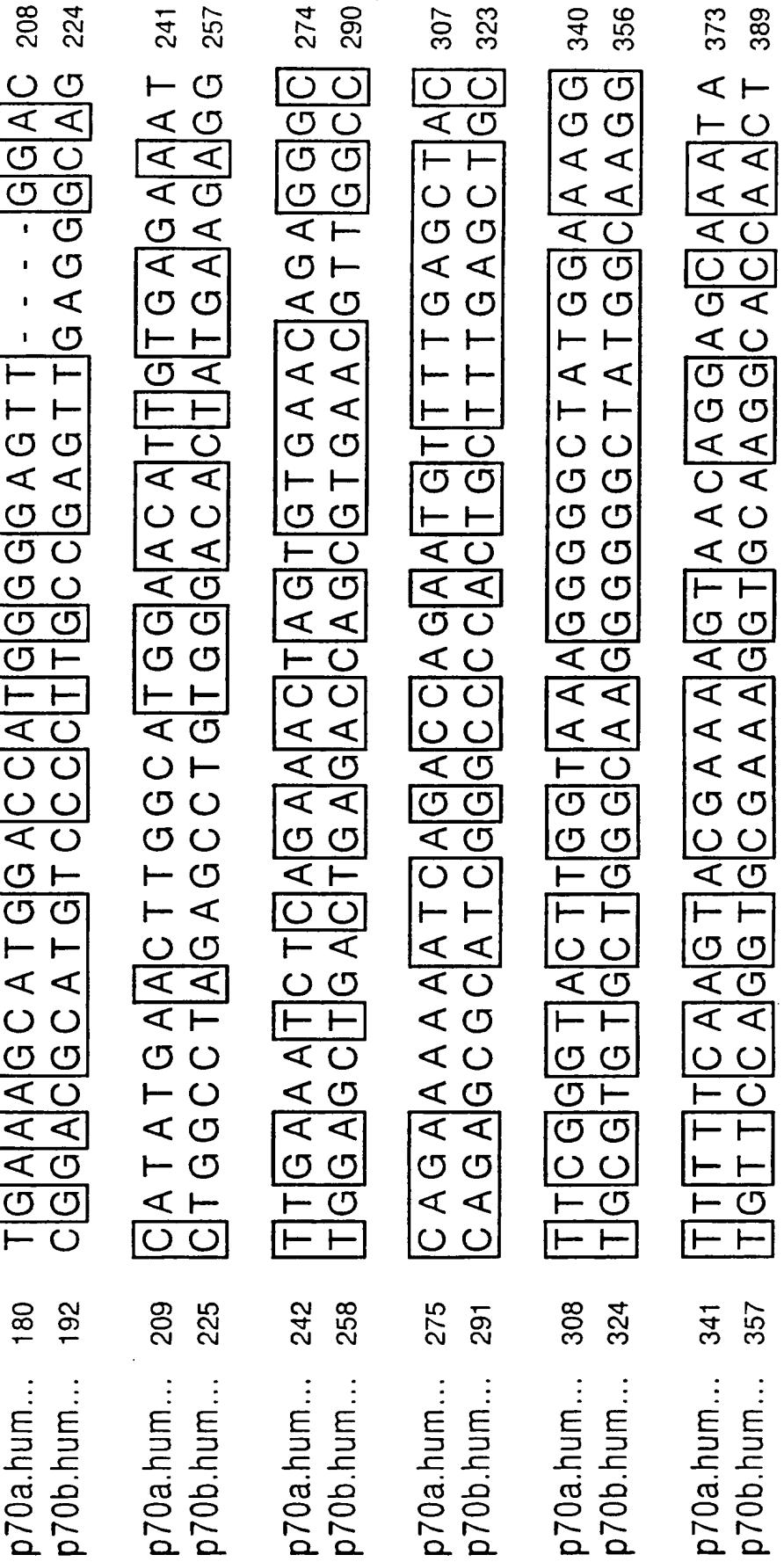

p70a.hum... 180 T G A A A G C A T G G A C C A T G G G G A G T T - - - - G G A C 208
p70b.hum... 192 C G G A C G C A T G T C C C C T T G C C G A G T T G A G G G C A G 224
p70a.hum... 209 C A T A T G A A C T T G G C A T G G A A C A T T G T G A G A A A T 241
p70b.hum... 225 C T G G C C T A G A G C C T G T G G G A C A C T A T G A A G G G 257
p70a.hum... 242 T T G A A A T C T C A G A A A C T A G T G T G A A C A G A G G G C 274
p70b.hum... 258 T G G A G C T G A C T G A G A C C A G C G T G A A C G T T G G C C 290
p70a.hum... 275 C A G A A A A A A T C A G A C C A G A A A T G T T T T G A G C T A C 307
p70b.hum... 291 C A G A G C G C A T C G G G C C C C A C T G C T T T G A G C T G C 323
p70a.hum... 308 T T C G G G T A C T T G G T A A A G G G G G C T A T G G A A A G G 340
p70b.hum... 324 T G C G T G T G C T G G G C A A G G G G G C T A T G G C A A G G 356
p70a.hum... 341 T T T T T C A A G T A C G A A A A G T A A C A G G A G C A A A T A 373
p70b.hum... 357 T G T T C C A G G T G C G A A A G G T G C A A G G C A C C A A C T 389

FIG. IC

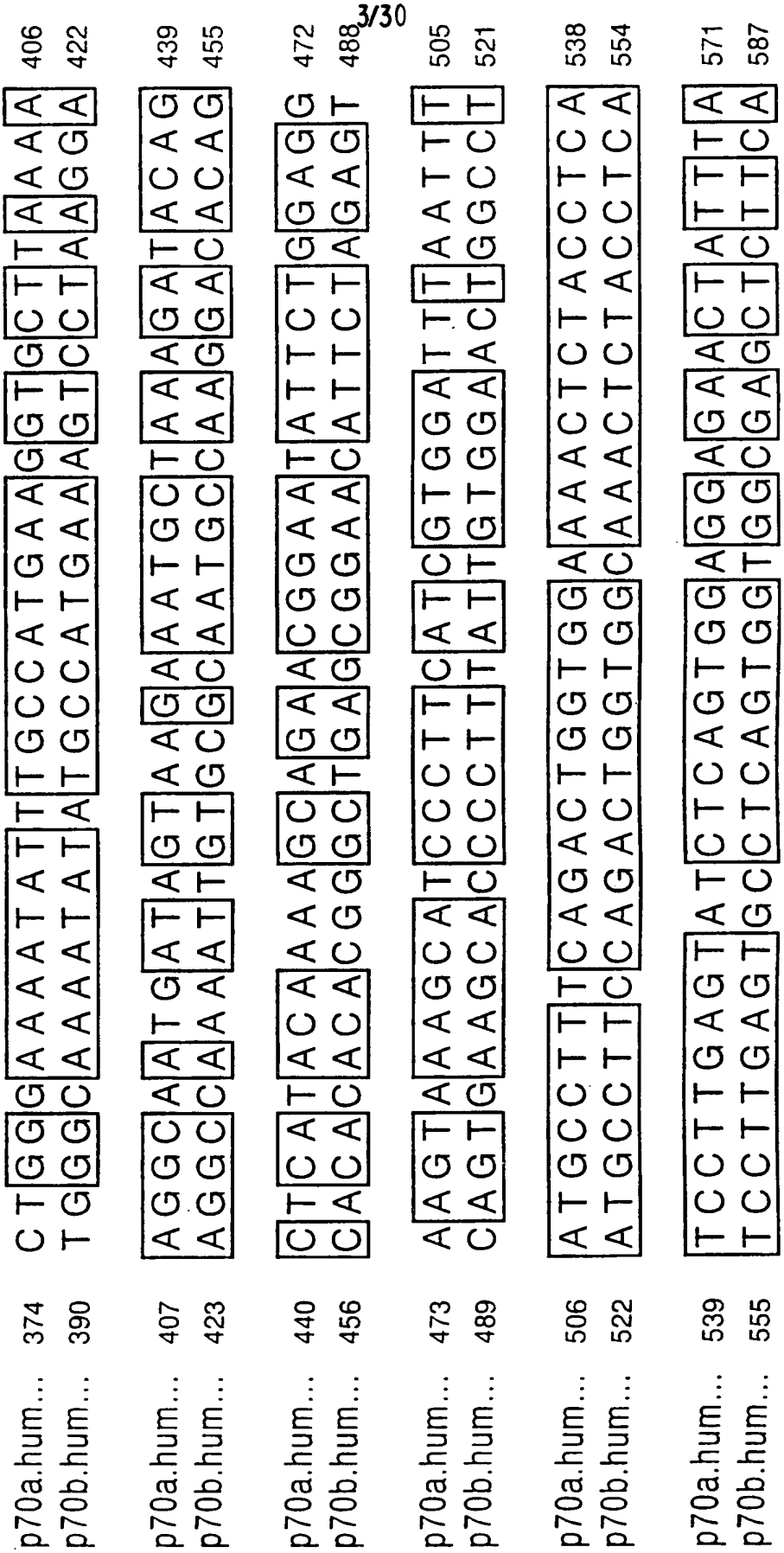

p70a.hum... 374 C T G G G A A A A T A T T T G C C A T G A A G G T G C T T A A A 406
p70b.hum... 390 T G G G C A A A A T A T A T G C C A T G A A A G T C C T A A G G A 422
p70a.hum... 407 A G G C A A T G A T A G T A A G A A A T G C T A A A G A T A C A G 439
p70b.hum... 423 A G G C C A A A T T G T G C G C A A T G C C A A G G A C A C A G 455
p70a.hum... 440 C T C A T A C A A A A G C A G A A C G G G A A T A T T C T G G A G G 472
p70b.hum... 456 C A C A C A C G G G C T G A G C G G A A C A T T C T A G A G T 488
p70a.hum... 473 A A G T A A A G C A T C C C T T C A T C G T G G A T T T A A T T T 505
p70b.hum... 489 C A G T G A A G C A C C C T T T A T T G T G G A A C T G G C C T 521
p70a.hum... 506 A T G C C T T T C A G A C T T G G T G G A A A A C T C T A C C T C A 538
p70b.hum... 522 A T G C C T T C C A G A C T G G T G G C A A A C T C T A C C T C A 554
p70a.hum... 539 T C C T T G A G T A T C T C A G T G G A G G A G A A C T A T T T A 571
p70b.hum... 555 T C C T T G A G T G C C T C A G T G G T G G C G A G C T C T T C A 587

FIG. 1D

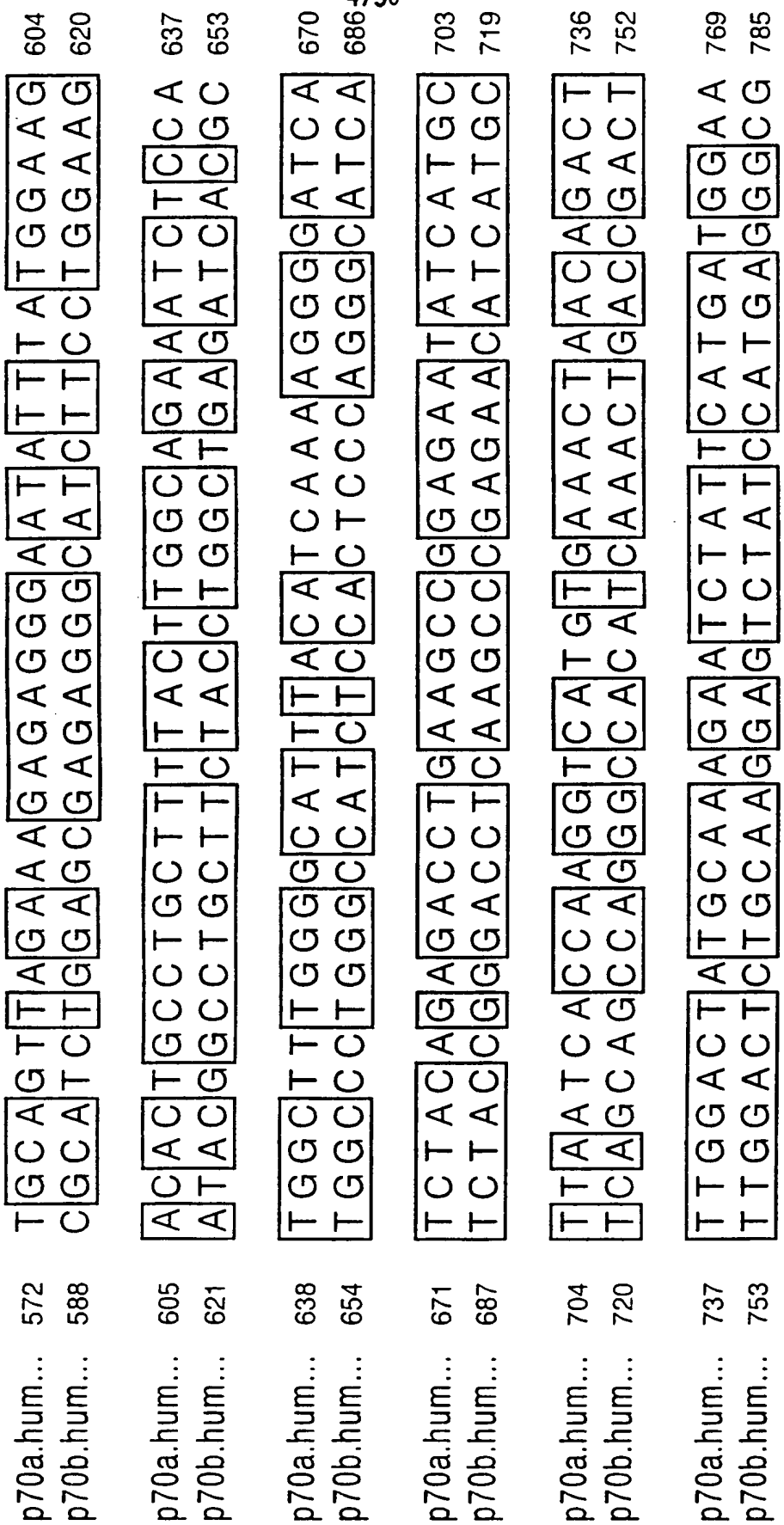

p70a.hum... 572 TGCAGTTAGAAAGAGAGGGAATATTTATGGAAG 604
p70b.hum... 588 CGCATCTGGAGCGAGAGGGCATCTTCCTGGAAG 620
p70a.hum... 605 ACACTGCCTGCTTTTACTGGCAGAATCTCCA 637
p70b.hum... 621 ATACGGCCTGCTTCTACCTGGCTGAGATCACGC 653
p70a.hum... 638 TGGCTTTGGGGCATTTTACATCAAAAGGGGGATCA 670
p70b.hum... 654 TGGCCCTGGGCCATCTCCACTCCCAGGGCATCA 686
p70a.hum... 671 TCTACAGAGACCTGAAGCCGGAGAATATCATGC 703
p70b.hum... 687 TCTACGGGACCTCAAGCCCGAGAACATCATGC 719
p70a.hum... 704 TTATCACCAAGGTCATGTTGAAACTAACAGACT 736
p70b.hum... 720 TCAGCAGGGCCACATCAAACTGACCGACT 752
p70a.hum... 737 TTGGACTATGCAAAGAAATCTATTTCATGATGGAA 769
p70b.hum... 753 TTGGACTCTGCAAGGAGTCTATCCATGAGGGCG 785

FIG. IE

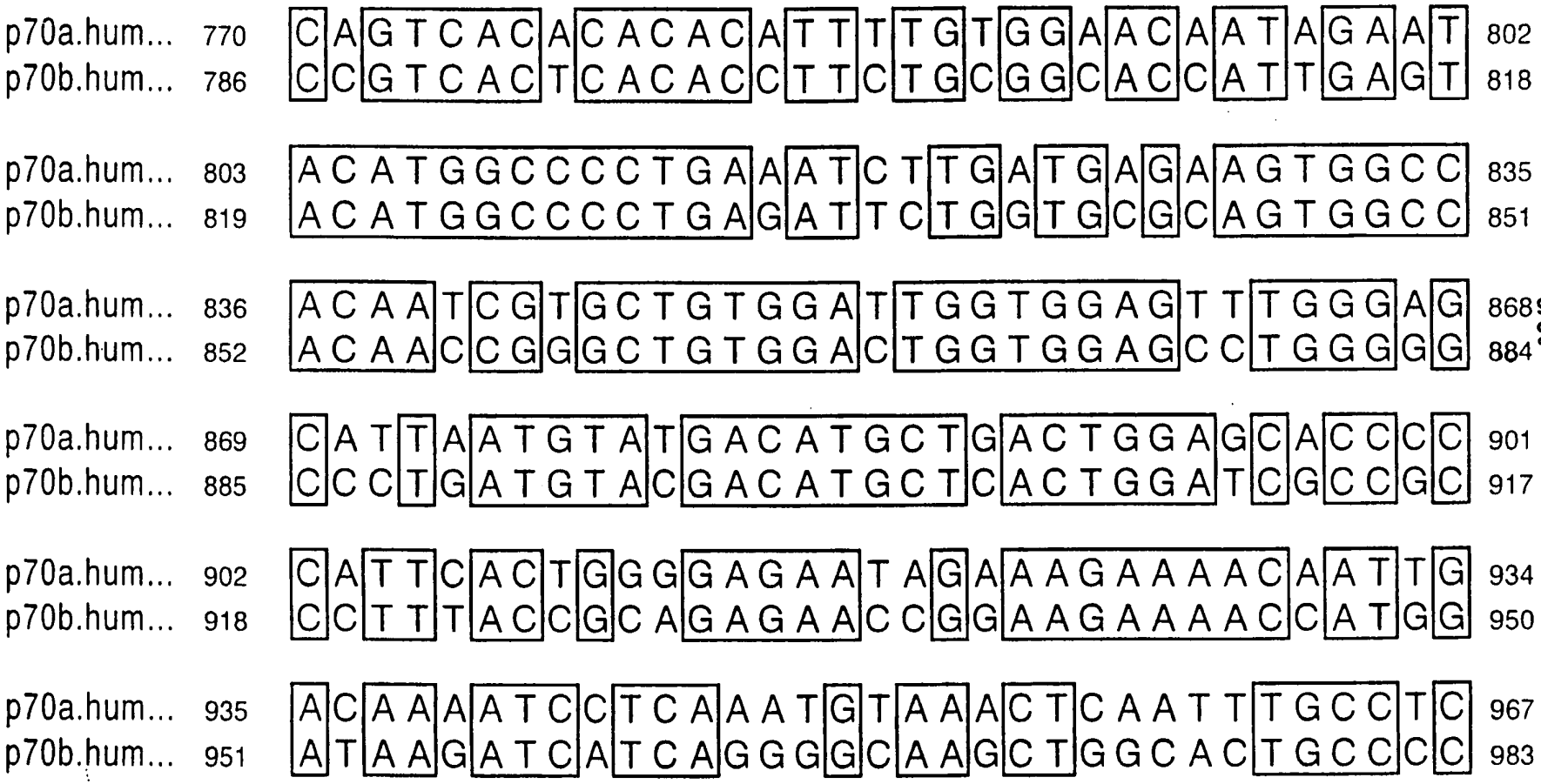


FIG. 1F

FIG. 1G

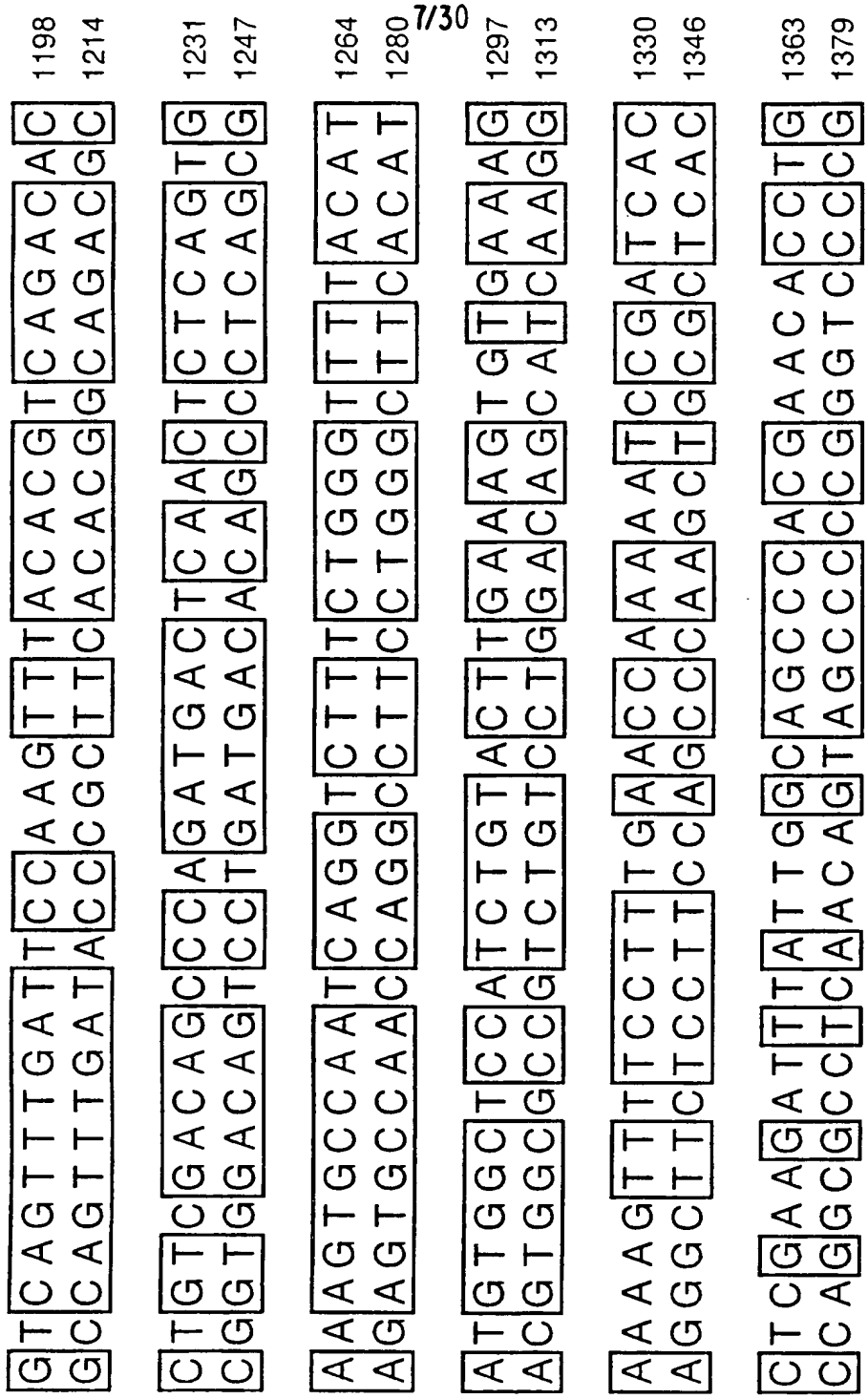

p70a.hum... 1166
p70b.hum... 1182
1198
1214
p70a.hum... 1199
p70b.hum... 1215
1231
1247
p70a.hum... 1232
p70b.hum... 1248
1264
1280
p70a.hum... 1265
p70b.hum... 1281
1297
1313
p70a.hum... 1298
p70b.hum... 1314
1330
1346
p70a.hum... 1331
p70b.hum... 1347
1363
1379

FIG. 1H

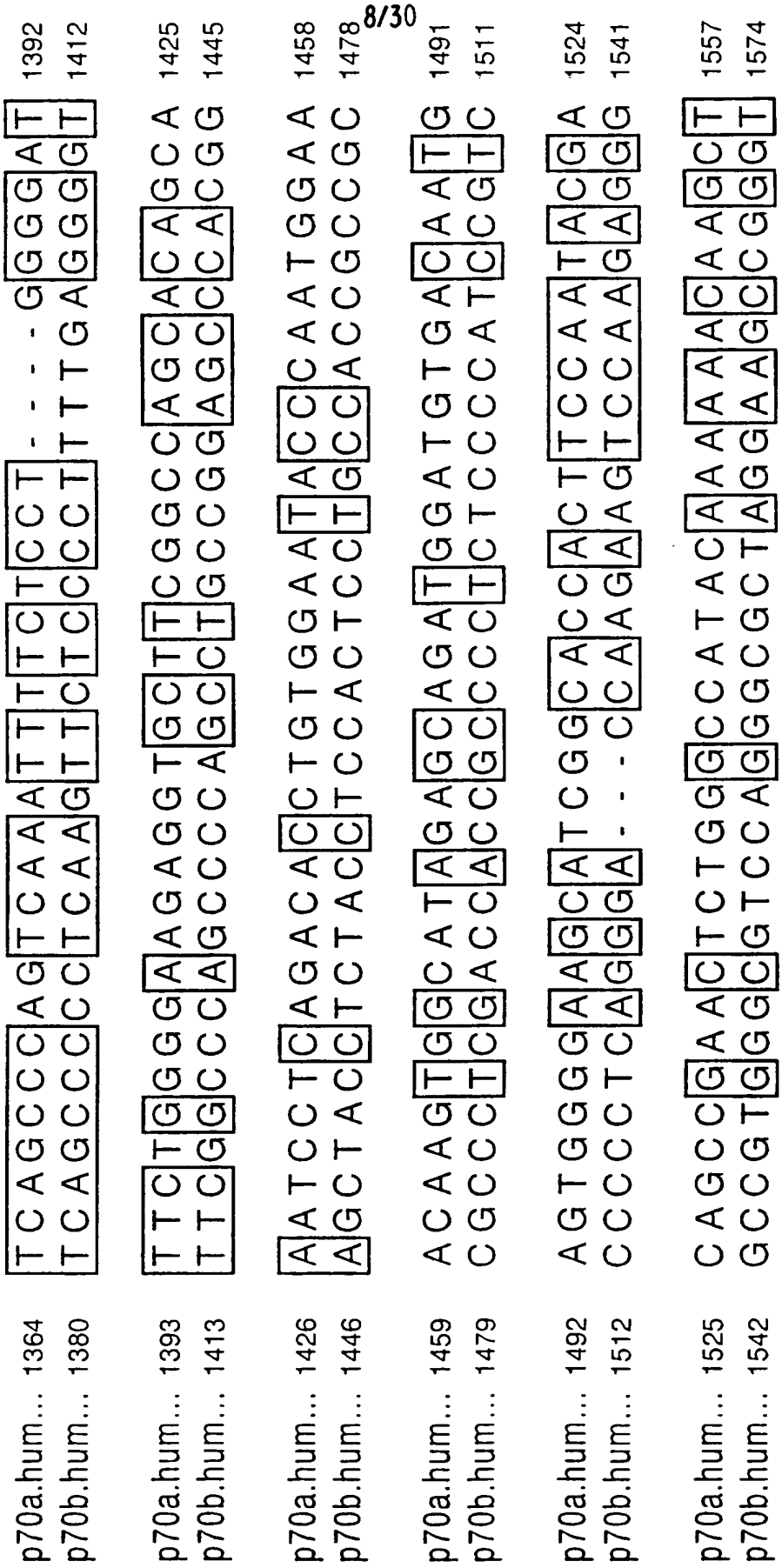

p70a.hum... 1364
p70b.hum... 1380
p70a.hum... 1393
p70b.hum... 1413
p70a.hum... 1426
p70b.hum... 1446
p70a.hum... 1459
p70b.hum... 1479
p70a.hum... 1492
p70b.hum... 1512
p70a.hum... 1525
p70b.hum... 1542
1392
1412
1425
1445
1458
1478
1491
1511
1524
1541
1557
1574

FIG. 11

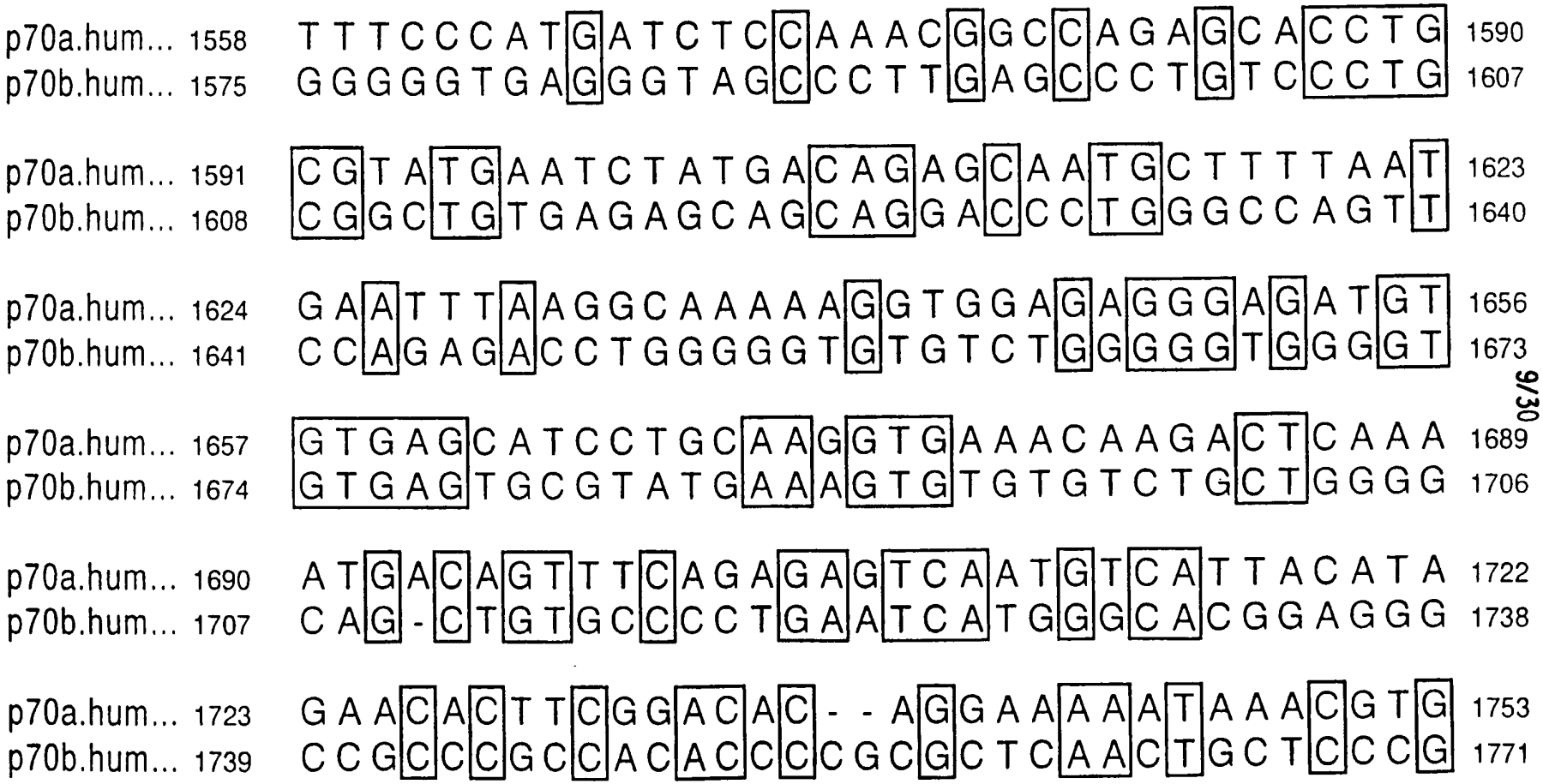


FIG. IJ

FIG. IK

p70a.hum... 1952 AGGGTATCCTTTCATTAGGCAAGTACAAATTGC 1984

p70a.hum... 1985 CTATAATACTTGCAACTAAGGACAAATTAGCAT 2017

p70a.hum... 2018 GCAAGCTTGGTCAAACTTTTCCCAGGCAAAATG 2050

p70a.hum... 2051 GGAAGGCAAAGACAAAAGAAACTTACCAATTGA 2083

p70a.hum... 2084 TGTTTTACGTGCAAACAACCTGAATCTTTTTTTT 2116

p70a.hum... 2117 TATATAAATATATATTTTTCAAATAGATTTTTG 2149

FIG. IL

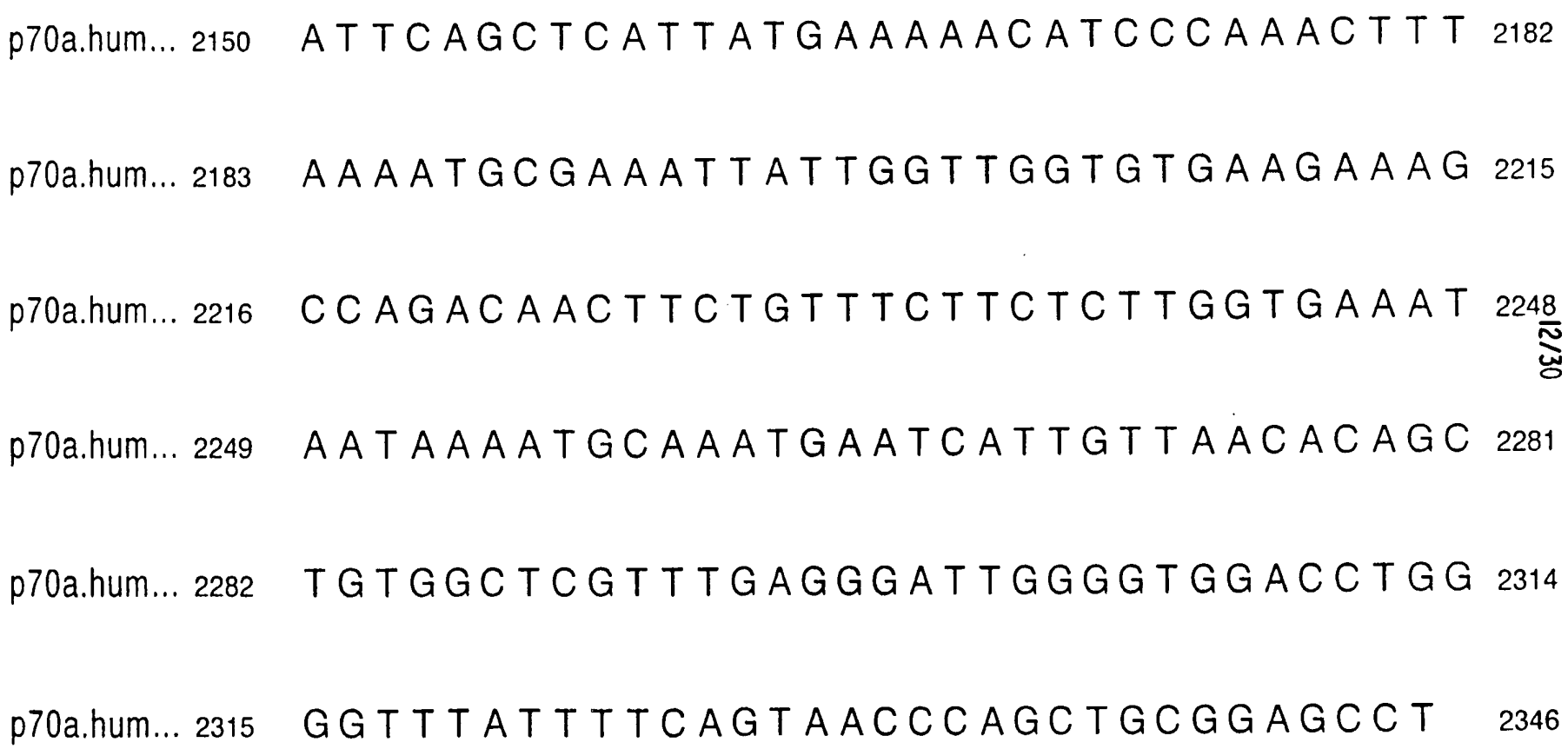


FIG. 2A-1

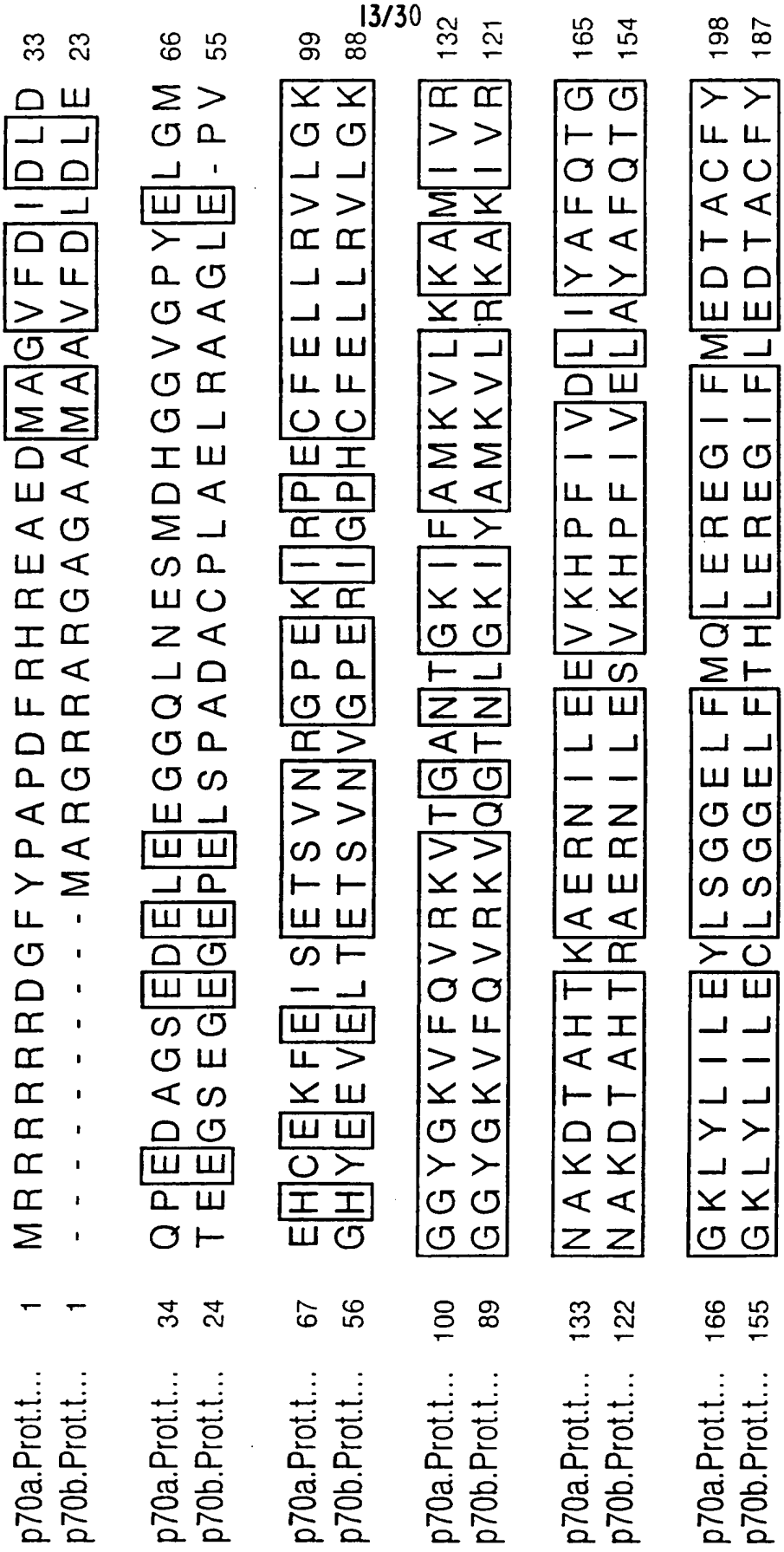

p70a.Prot.t... 1 M R R R R R D G F Y P A P D F R H R E A E D M A G V F D I D L D 33
p70b.Prot.t... 1 - - - - - - - - - - M A R G R R A R G A G A A M A A V F D L D L E 23
p70a.Prot.t... 34 Q P E D A G S E D E L E E G G Q L N E S M D H G G V G P Y E L G M 66
p70b.Prot.t... 24 T E E G S E G E G E P E L S P A D A C P L A E L R A A G L E - P V 55
p70a.Prot.t... 67 E H C E K F E I S E T S V N R G P E K I R P E C F E L L R V L G K 99
p70b.Prot.t... 56 G H Y E E V E L T E T S V N V G P E R I G P H C F E L L R V L G K 88
p70a.Prot.t... 100 G G Y G K V F Q V R K V T G A N T G K I F A M K V L K K A M I V R 132
p70b.Prot.t... 89 G G Y G K V F Q V R K V Q G T N L G K I Y A M K V L R K A K I V R 121
p70a.Prot.t... 133 N A K D T A H T K A E R N I L E E V K H P F I V D L I Y A F Q T G 165
p70b.Prot.t... 122 N A K D T A H T R A E R N I L E S V K H P F I V E L A Y A F Q T G 154
p70a.Prot.t... 166 G K L Y L I L E Y L S G G E L F M Q L E R E G I F M E D T A C F Y 198
p70b.Prot.t... 155 G K L Y L I L E C L S G G E L F T H L E R E G I F L E D T A C F Y 187

FIG. 2A-2

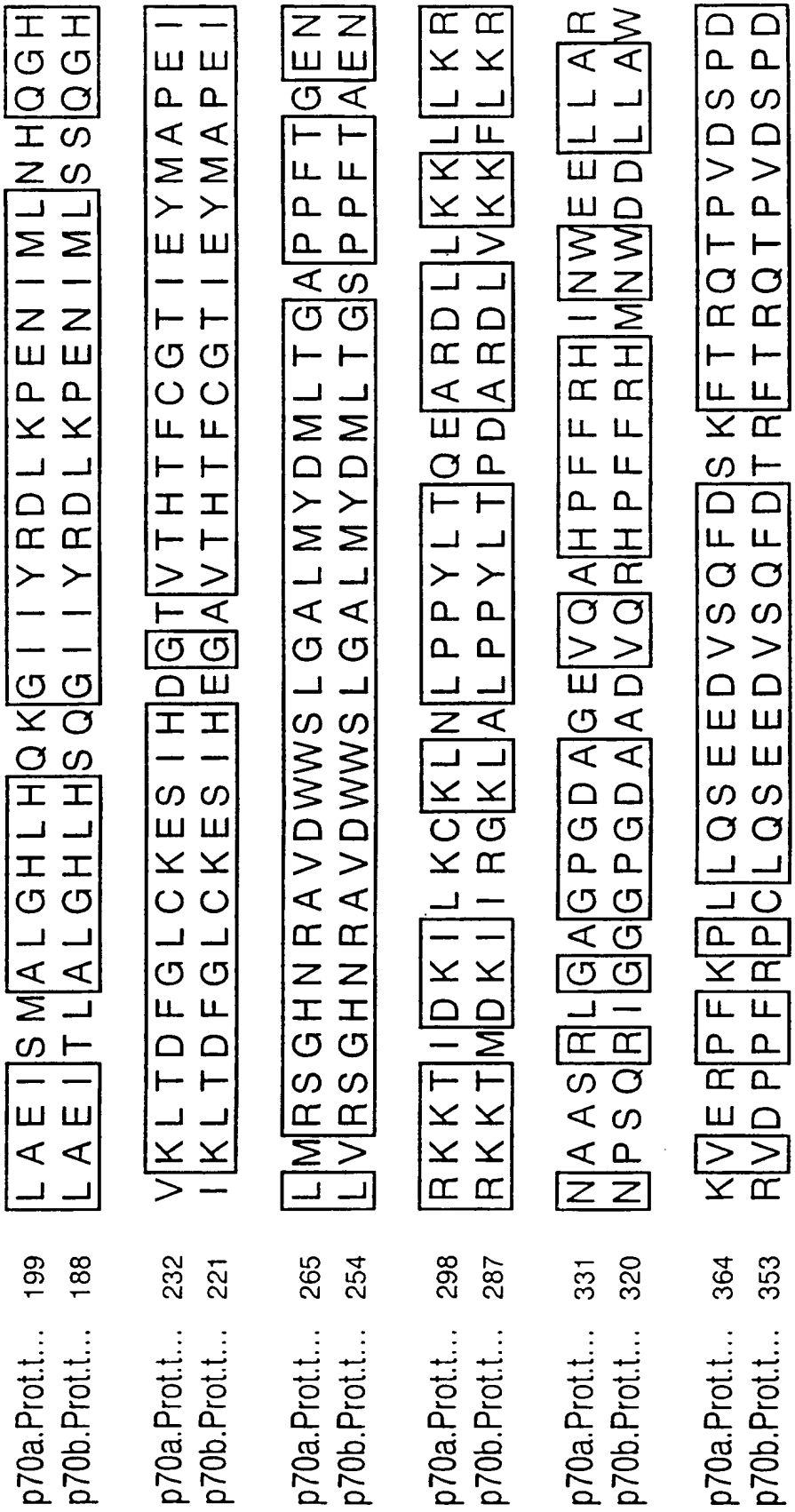
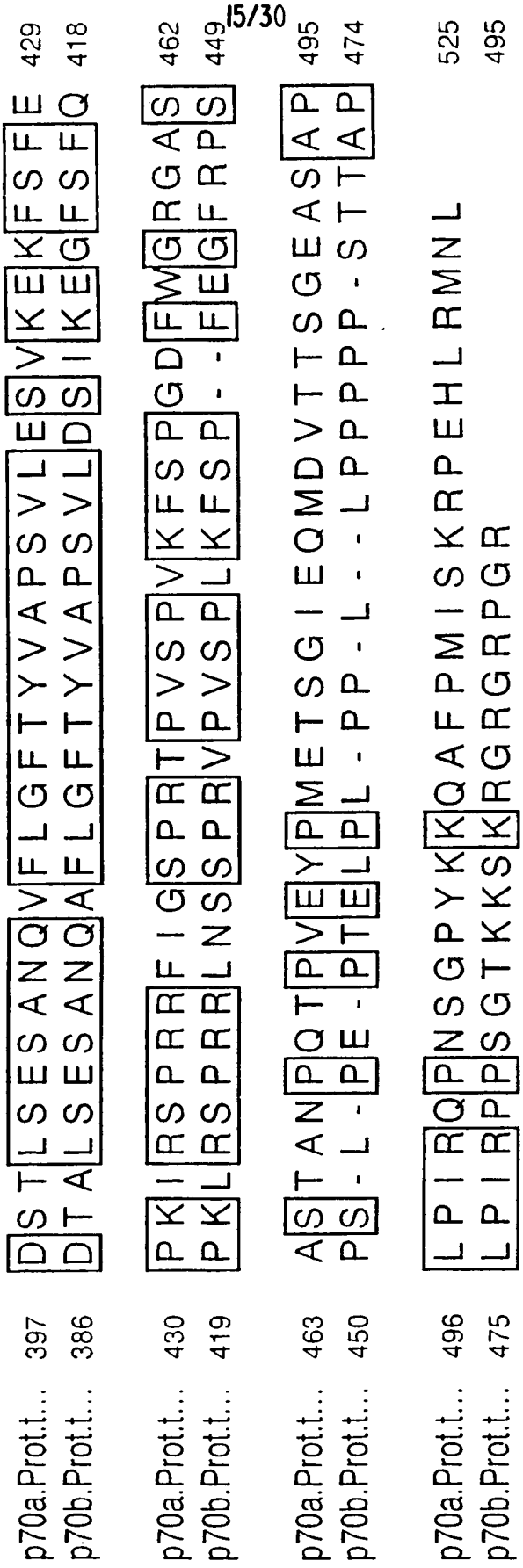

p70a.Prot.t... 199 LAEISMALGHLHQKGIIYRDLKPENIMLNHQGH 231
p70b.Prot.t... 188 LAEITLALGHLHSQGIIYRDLKPENIMLSSQGH 220
p70a.Prot.t... 232 VKLTDFGLCKESIHDGTVTHTFCGTIEYMAPEI 264
p70b.Prot.t... 221 IKLTDFGLCKESIHEGAVTHTFCGTIEYMAPEI 253
p70a.Prot.t... 265 LMRSGHNRAVDWWSLGALMYDMLTGAPPFTGEN 297
p70b.Prot.t... 254 LVRSGHNRAVDWWSLGALMYDMLTGSPPFTAEN 286
p70a.Prot.t... 298 RKKTIDKILKCKLNLPPYLTQEARDLLKKLLKR 330
p70b.Prot.t... 287 RKKTMDKIIRGKLALPPYLTPDARDLVKKFLKR 319
p70a.Prot.t... 331 NAASRLGAGPGDAGEVQAHPFFRHINWEELLAR 363
p70b.Prot.t... 320 NPSQRIGGGPGDADVQRHPFFRHMNWDLLAAW 352
p70a.Prot.t... 364 KVERPFKPLLQSEEDVSQFDSKFTRQTPVDSPD 396
p70b.Prot.t... 353 RVDPPFRPCLQSEEDVSQFDTRFTRQTPVDSPD 385

FIG. 2A-3


p70a.Prot.t... 397 DSTLSESANQVFLGFTYVAPSVLESVKEKFSFE 429
p70b.Prot.t... 386 DTALSESANQAFLGFTYVAPSVLDSIKEGFSFQ 418
p70a.Prot.t... 430 PKIRSPRRFIGSPRTPVSPVKFSPGDFMGRGAS 462
p70b.Prot.t... 419 PKLRSPRRLNSSPRVPVSPLKFSP--FEGFRPS 449
p70a.Prot.t... 463 ASTANPQTPVEYPMETSGIEQMDVTTSGEASAP 495
p70b.Prot.t... 450 PS-L-PE-PTELPL-PP-L--LPPPPP-STTAP 474
p70a.Prot.t... 496 LPIRQPNSGPYKKQAFPMISKRPEHLRMNL 525
p70b.Prot.t... 475 LPIRPPSGTKKSKRGRGRPGR 495

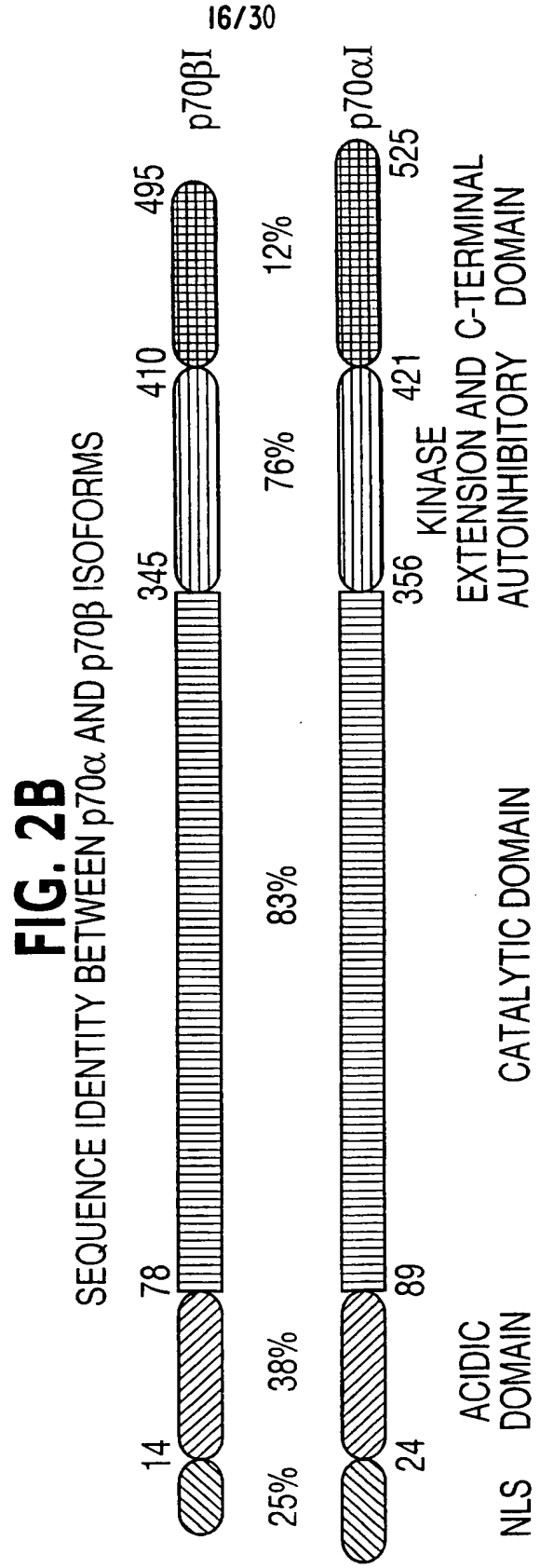


FIG. 2B
SEQUENCE IDENTITY BETWEEN p70α AND p70β ISOFORMS

FIG. 3A

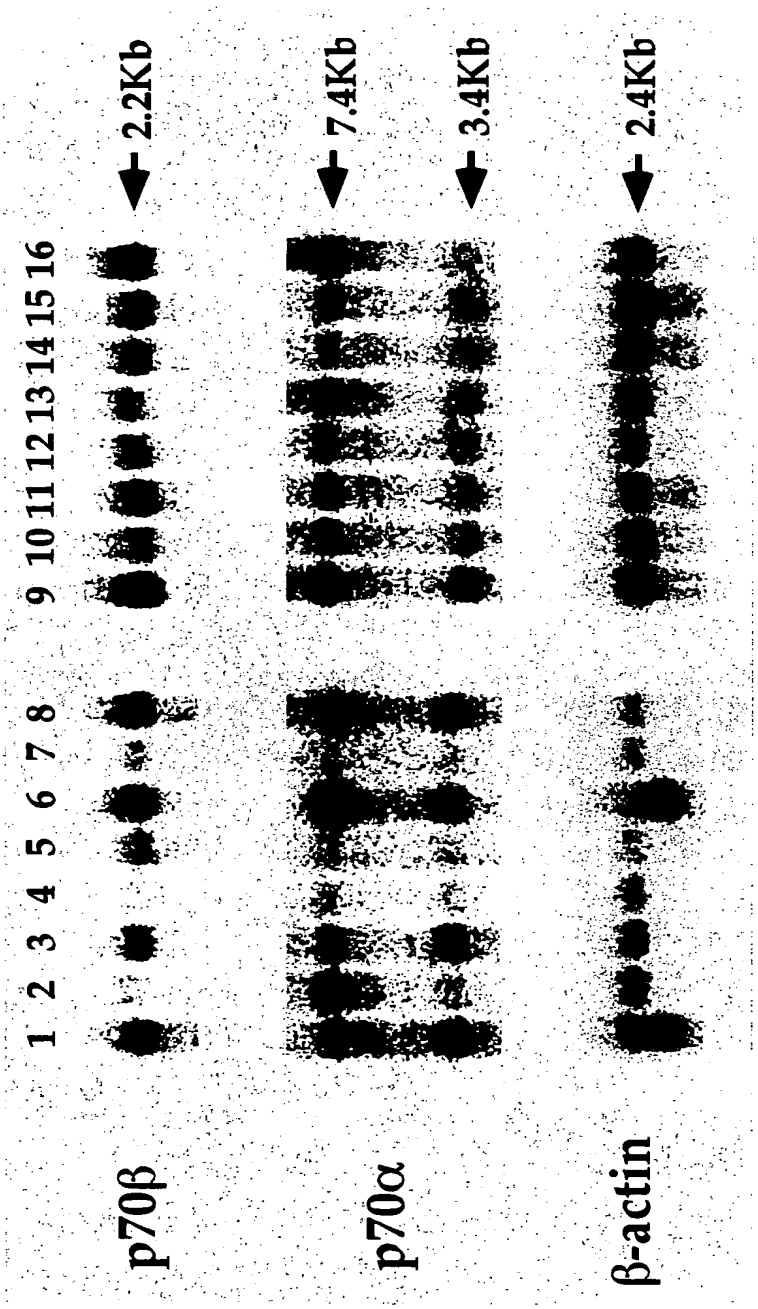
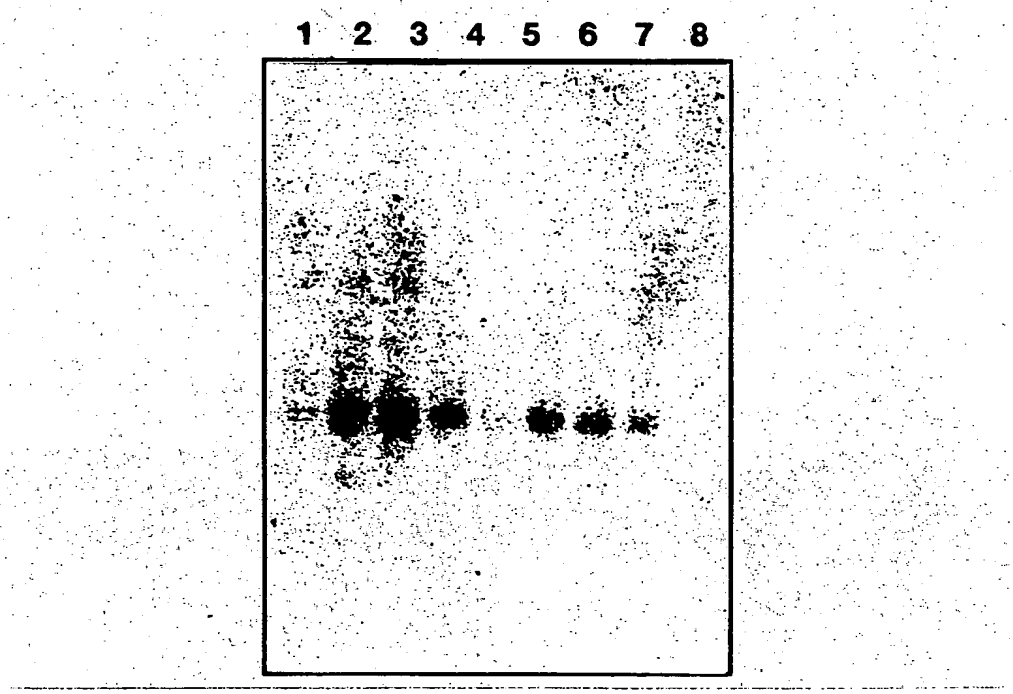

2.2Kb
7.4Kb
3.4Kb
2.4Kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
p70β
p70α
β-actin

FIG. 3B

Expression pattern of the p70β mRNAs in tumour cell lines



1 Promyelocytic leukemia HL-60
2 HeLa cell S3
3 chronic myelogenous leukemia K562
4 Lymphoblastic leukemia MOLT-4
5 Burkitt's lymphoma Raji
6 colorectal adenocarcinoma SW480
7 Lung carcinoma A549
8 Melanoma G361

FIG. 4A

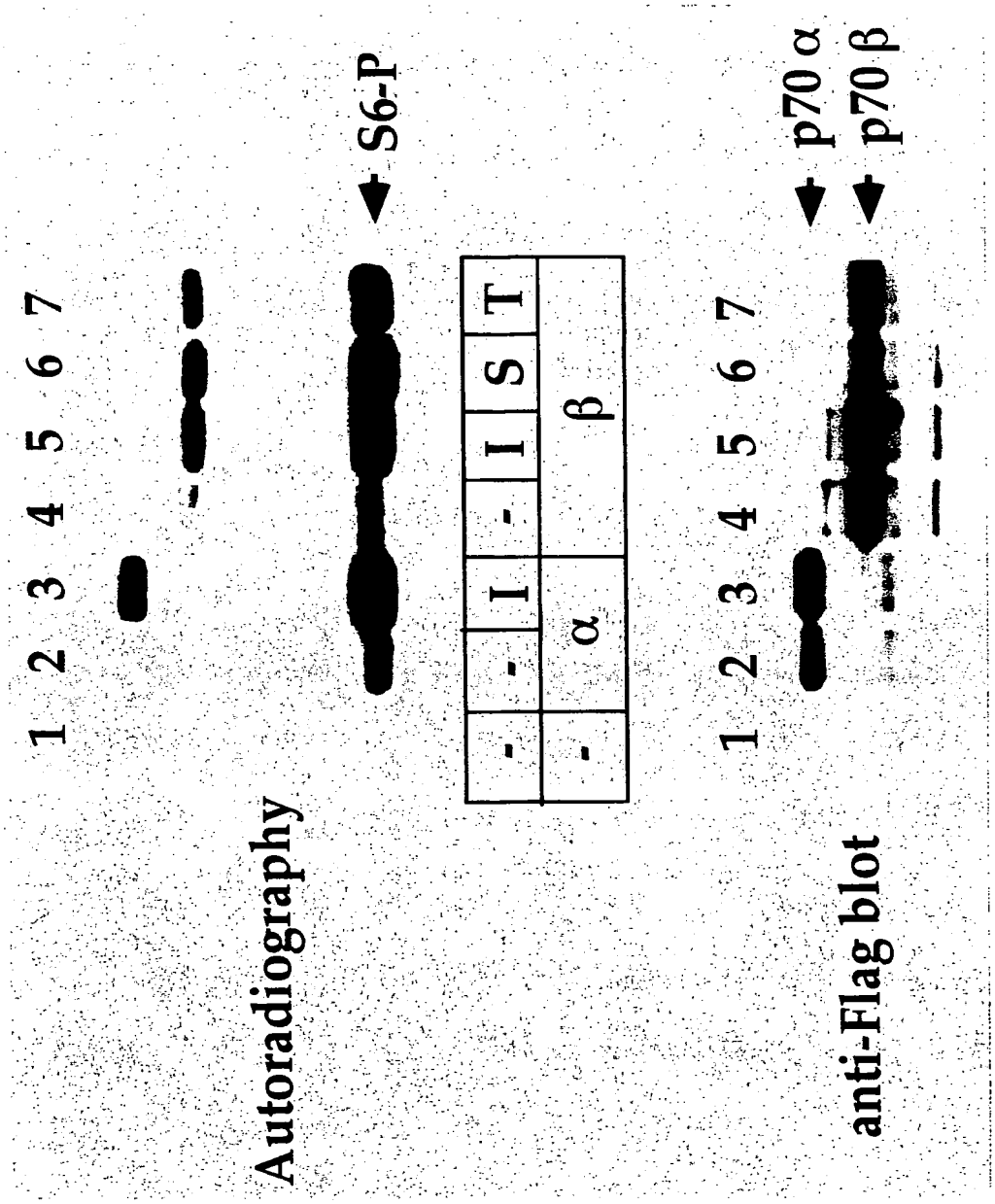

1 2 3 4 5 6 7
S6-P
Autoradiography
- - I - I S T
- α β
1 2 3 4 5 6 7
p70 α
p70 β
anti-Flag blot

FIG. 4B

PHOSPHORYLATION OF THE RIBOSOMAL S6 PROTEIN C-TERMINAL PEPTIDES BY p70α AND β KINASES

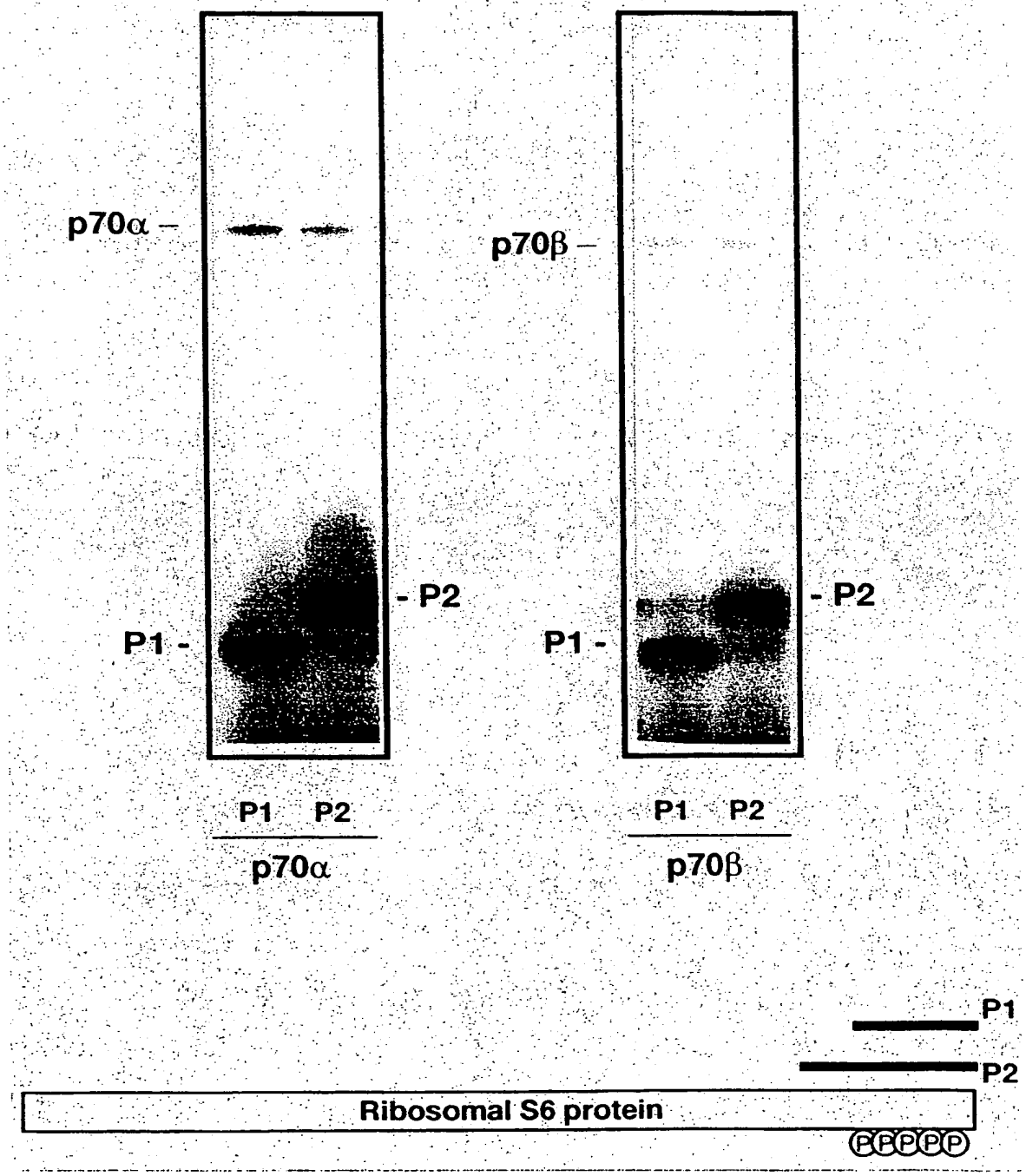
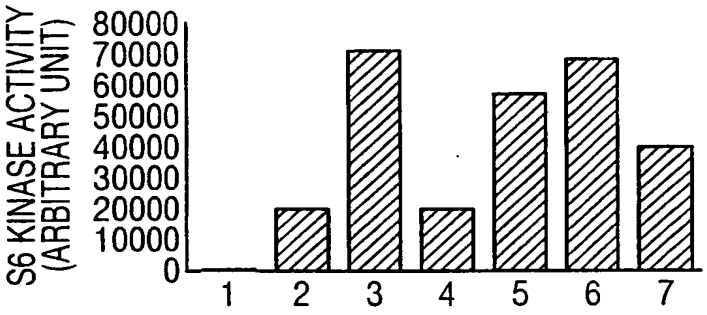


FIG. 5A

ACTIVATION OF THE P70α AND β KINASES IN RESPONSE TO VARIOUS STIMULI IN VIVO



1 MOCK TRANSFECTION
2 p70α (STARVED AND NONTREATED)
3 p70α (STARVED AND INSULIN STIMULATED)
4 p70β (STARVED AND NONTREATED)
5 p70β (STARVED AND INSULIN STIMULATED)
6 p70β (STARVED AND SERUM STIMULATED)
7 p70β (STARVED AND TPA STIMULATED)

FIG. 5B

ACTIVATION OF THE P70α AND β KINASES BY PDGF IN TRANSIENTLY TRANSFECTED PAE CELLS

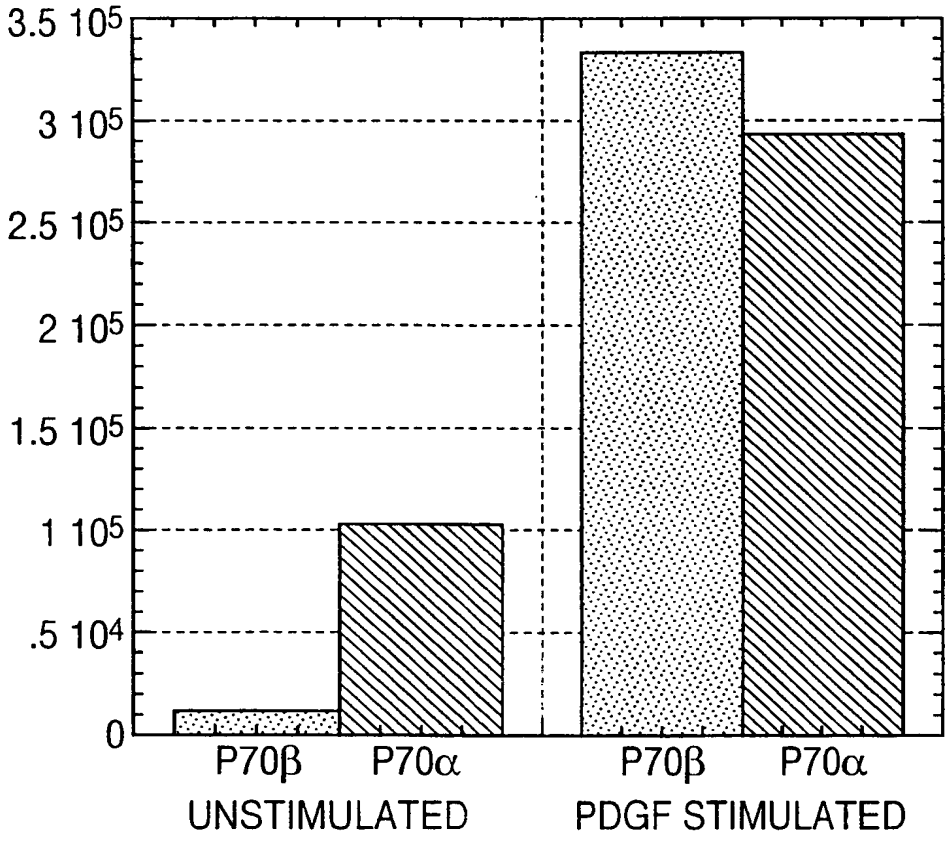


FIG. 6A

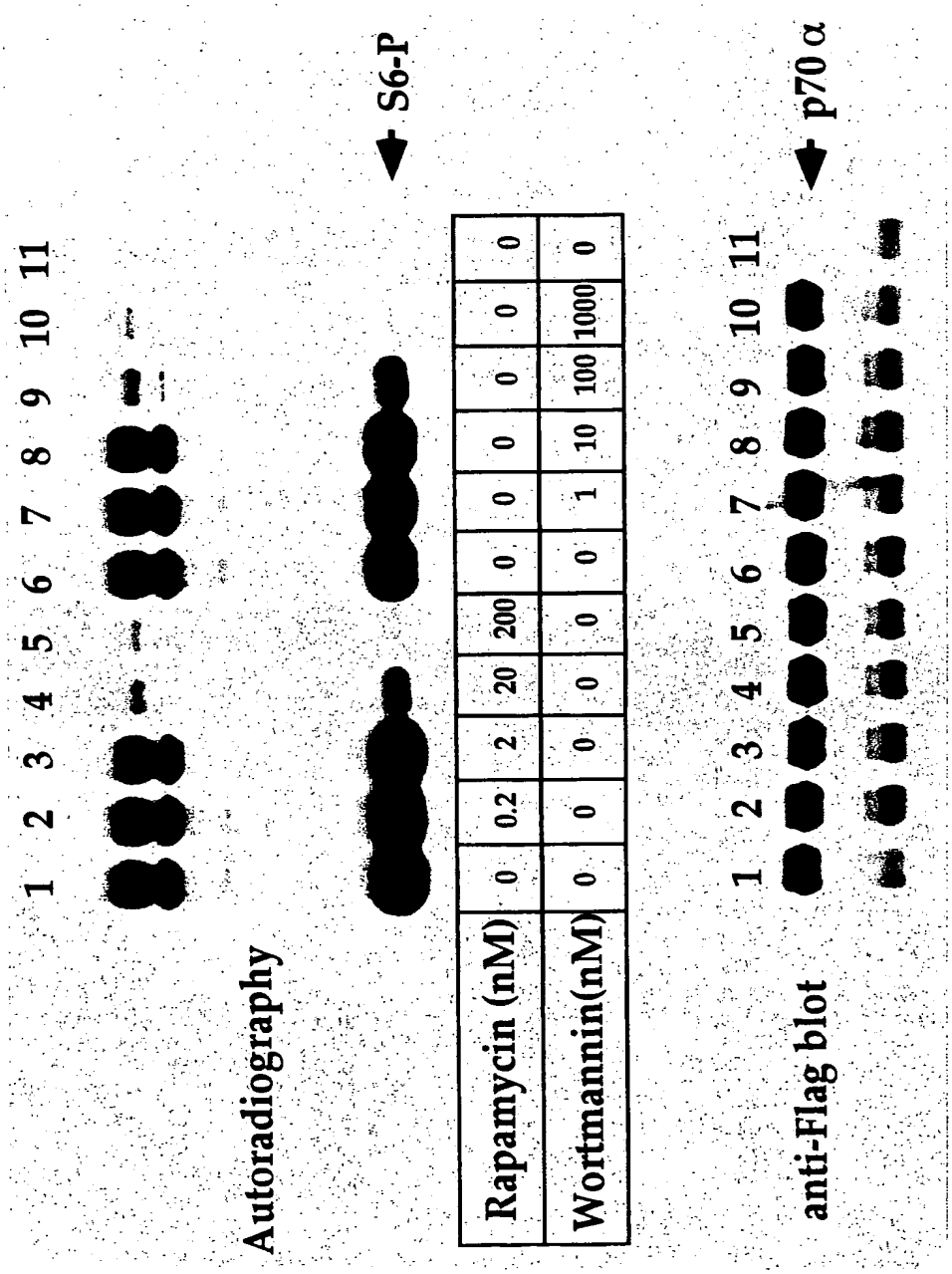

1 2 3 4 5 6 7 8 9 10 11
Autoradiography
S6-P
Rapamycin (nM) 0 0.2 2 20 200 0 0 0 0 0 0
Wortmannin(nM) 0 0 0 0 0 0 1 10 100 1000 0
1 2 3 4 5 6 7 8 9 10 11
anti-Flag blot
p70 α

FIG. 6B

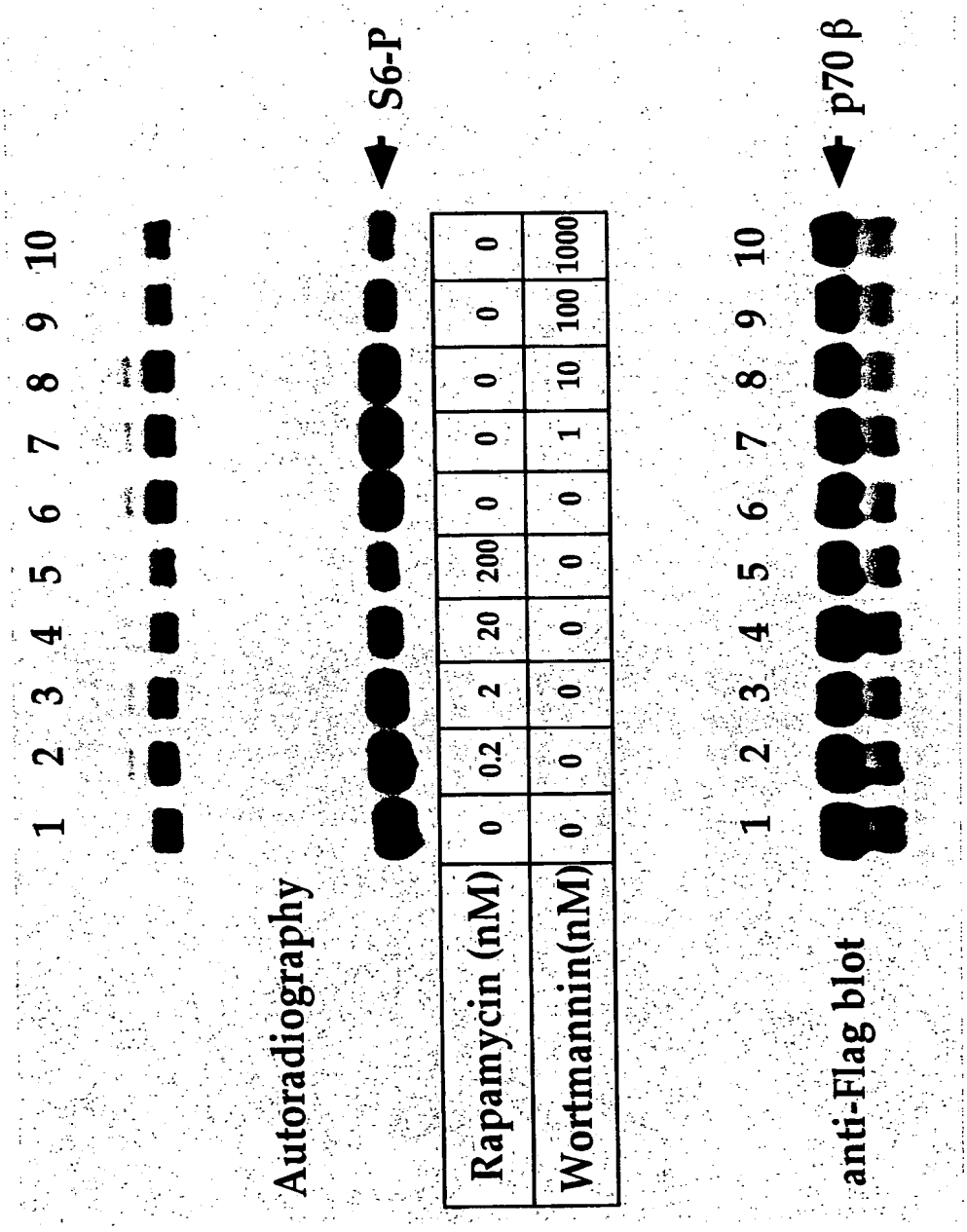

1 2 3 4 5 6 7 8 9 10
Autoradiography
S6-P
Rapamycin (nM) 0 0.2 2 20 200 0 0 0 0 0
Wortmannin(nM) 0 0 0 0 0 0 1 10 100 1000
1 2 3 4 5 6 7 8 9 10
anti-Flag blot
p70 β

FIG. 7

INTERACTION OF P70S6K β WITH DIFFERENT GST/SH3 FUSION PROTEINS IN VITRO

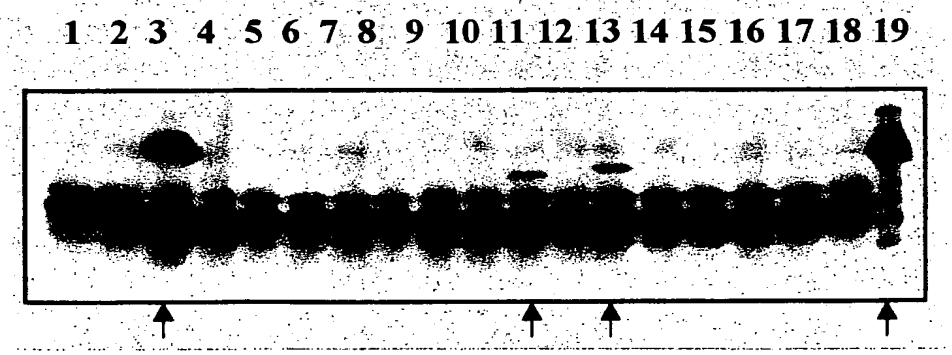
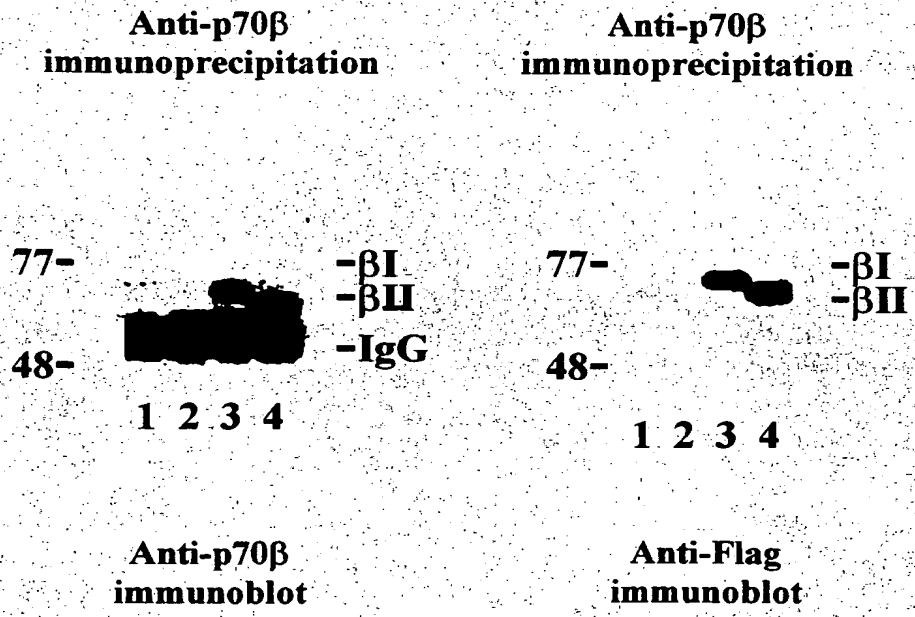


FIG. 8

IMMUNOPRECIPATATION AND WESTERN BLOT ANALYSIS OF P70βI AND βII TRANSIENTLY OVEREXPRESSED IN HEK 293 CELLS



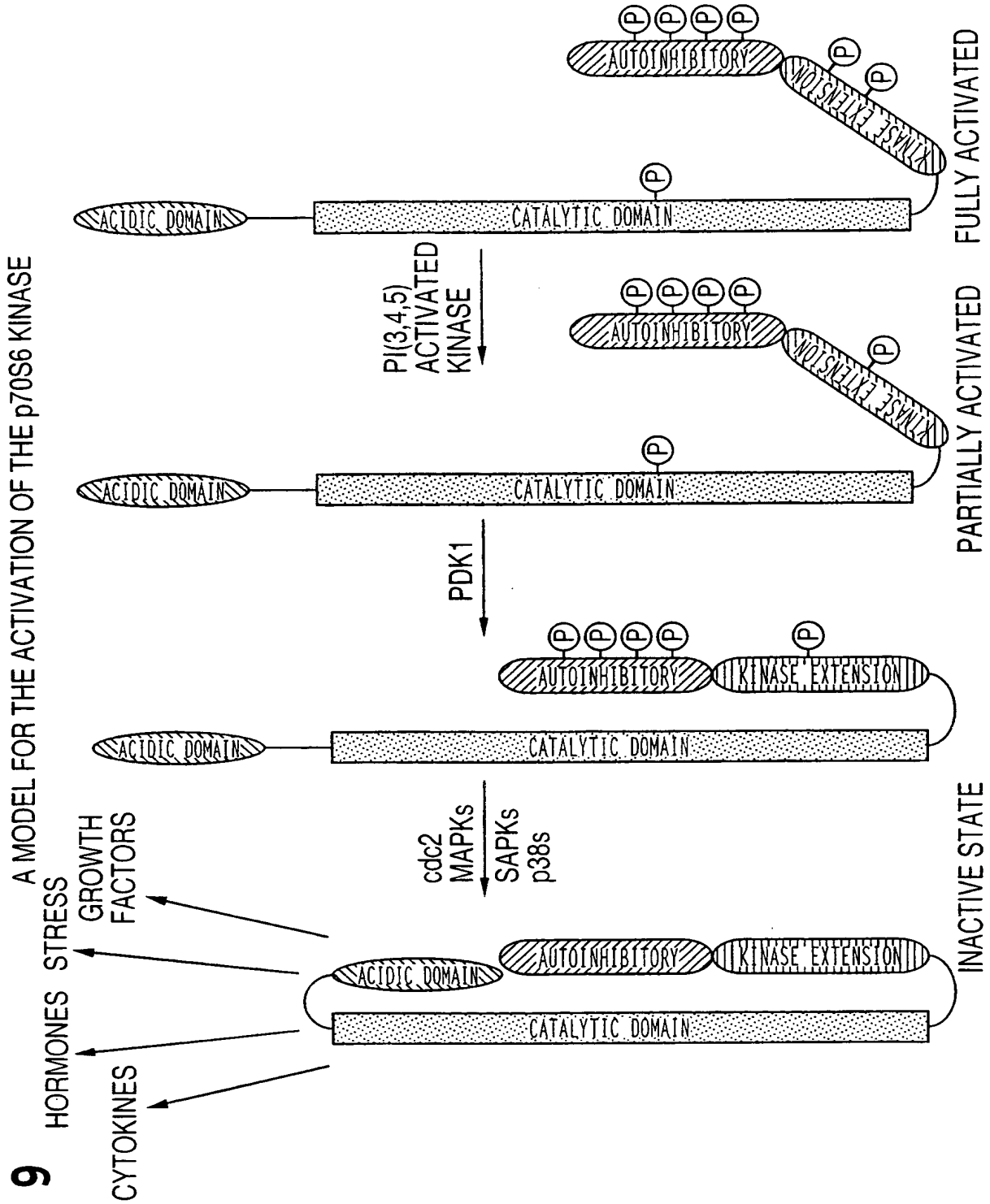

A MODEL FOR THE ACTIVATION OF THE p70S6 KINASE
AUTOINHIBITORY
KINASE EXTENSION
ACIDIC DOMAIN
CATALYTIC DOMAIN
FULLY ACTIVATED
PI(3,4,5) ACTIVATED KINASE
PARTIALLY ACTIVATED
PDK1
INACTIVE STATE
cdc2 MAPKs
SAPKs p38s
STRESS
GROWTH FACTORS
HORMONES
CYTOKINES

FIG. 9

FIG. 10

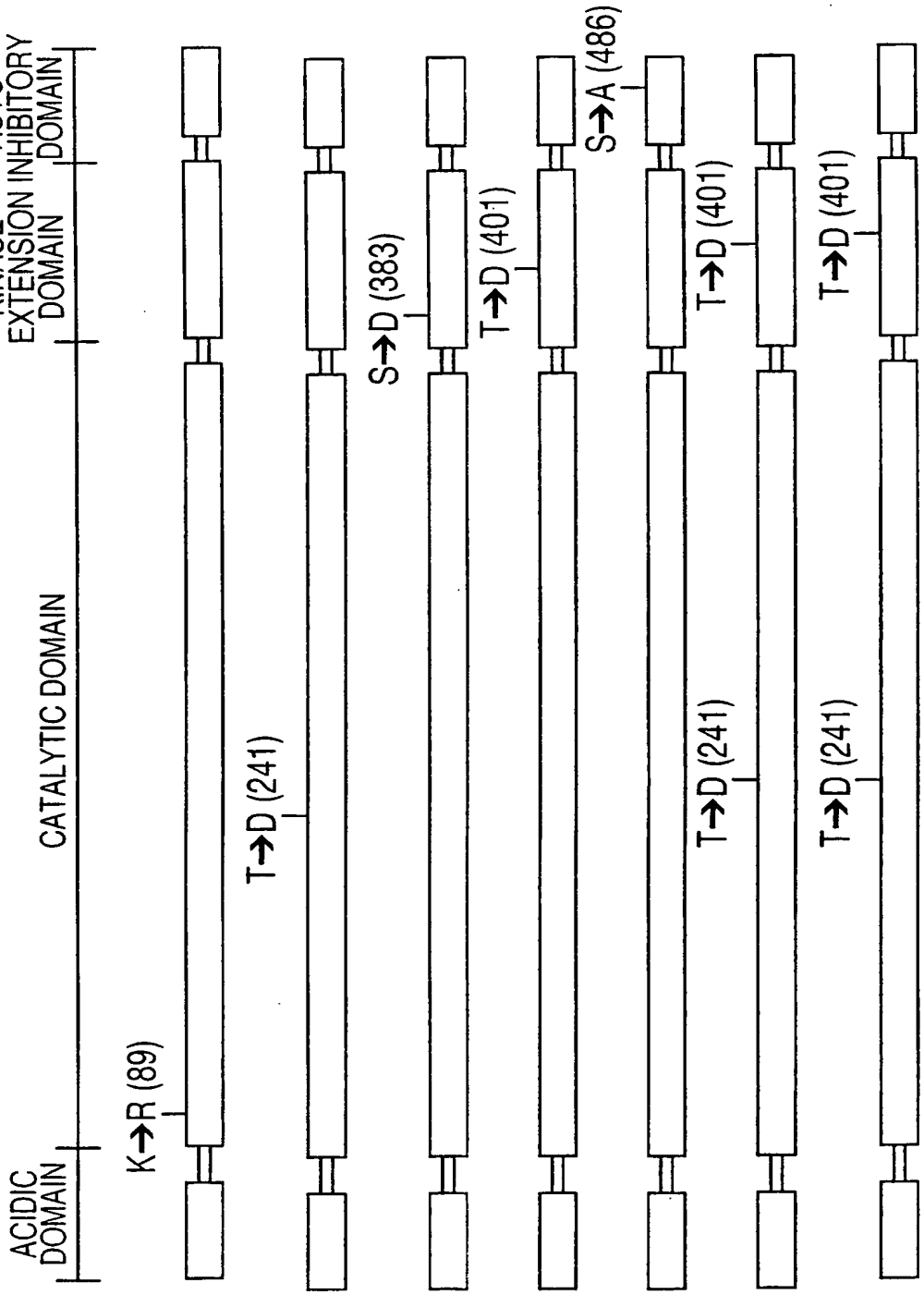

p70β MUTATIONS
ACIDIC DOMAIN
CATALYTIC DOMAIN
KINASE EXTENSION DOMAIN
AUTO-INHIBITORY DOMAIN
K→R (89)
T→D (241)
S→D (383)
T→D (401)
S→A (486)
T→D (401)
T→D (241)
T→D (401)
T→D (241)

FIG. 11

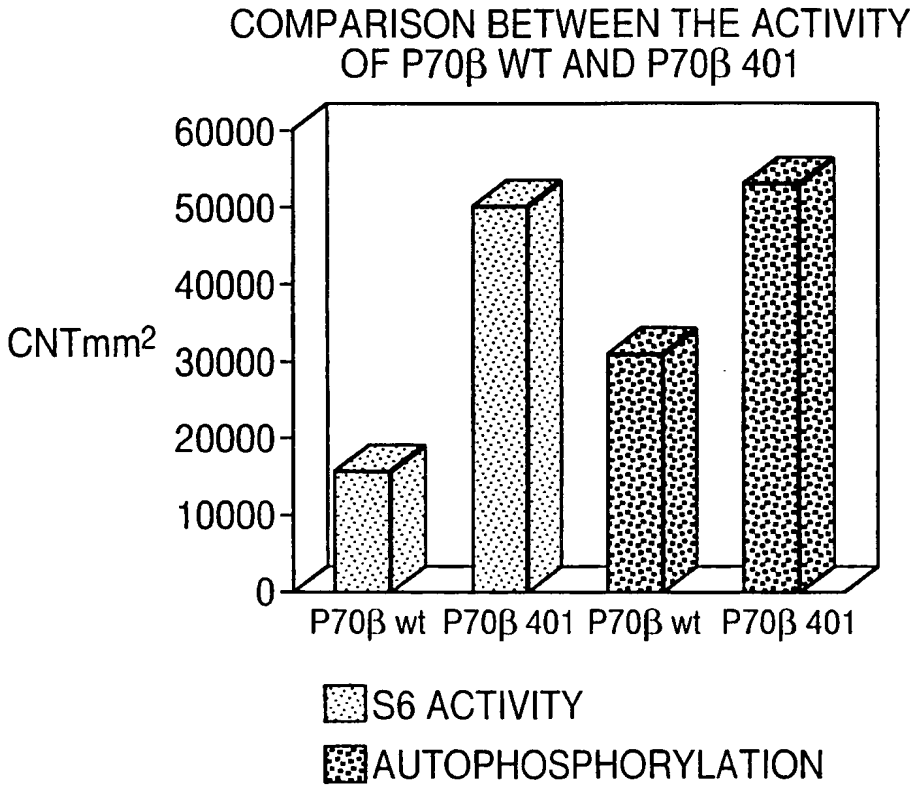
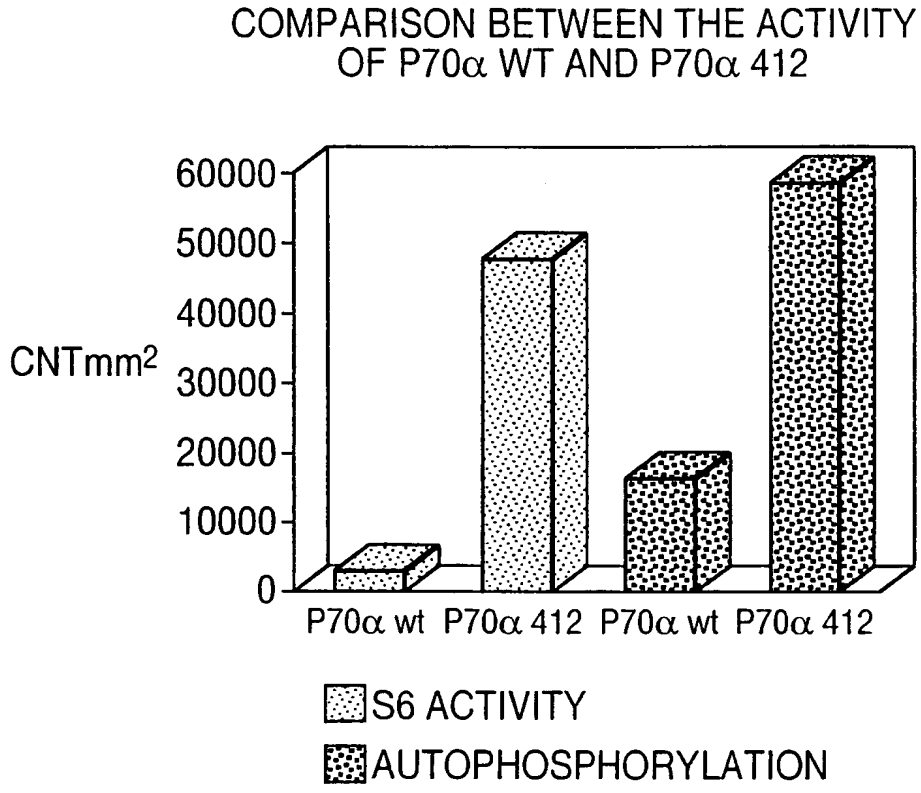

COMPARISON BETWEEN THE ACTIVITY
OF P70β WT AND P70β 401
CNTmm2
60000
50000
40000
30000
20000
10000
0
P70β wt
P70β 401
P70β wt
P70β 401
S6 ACTIVITY
AUTOPHOSPHORYLATION

FIG. 12


COMPARISON BETWEEN THE ACTIVITY
OF P70α WT AND P70α 412
CNTmm2
60000
50000
40000
30000
20000
10000
0
P70α wt
P70α 412
P70α wt
P70α 412
S6 ACTIVITY
AUTOPHOSPHORYLATION

SEQUENCE LISTING

<110> Ludwig Institute for Cancer Research

<110> Gout, Ivan
Hara, Kenta
Waterfield, Michael
Yonezawa, Kazu

<120> Identification and Functional Characterization of a Novel Ribosomal S6 Protein Kinase

<130> 40750-5002

<140> Unassigned
<141> 1999-08-04

<150> 60/095,268
<151> 1998-08-04

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<170> PatentIn Ver. 2.0

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<222> (77)..(1561)
<223> p70(beta) S6 Kinase gene

<400> 1
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Met Ala Arg Gly Arg Arg Ala Arg Gly Ala Gly Ala
1 5 10

gcc atg gcg gcc gtg ttt gat ttg gat ttg gag acg gag gaa ggc agc 160
Ala Met Ala Ala Val Phe Asp Leu Asp Leu Glu Thr Glu Glu Gly Ser
15 20 25

gag ggc gag ggc gag cca gag ctc agc ccc gcg gac gca tgt ccc ctt 208
Glu Gly Glu Gly Glu Pro Glu Leu Ser Pro Ala Asp Ala Cys Pro Leu
30 35 40

gcc gag ttg agg gca gct ggc cta gag cct gtg gga cac tat gaa gag 256
Ala Glu Leu Arg Ala Ala Gly Leu Glu Pro Val Gly His Tyr Glu Glu
45 50 55 60

gtg gag ctg act gag acc agc gtg aac gtt ggc cca gag cgc atc ggg 304
Val Glu Leu Thr Glu Thr Ser Val Asn Val Gly Pro Glu Arg Ile Gly
65 70 75

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Pro His Cys Phe Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly
             80                  85                  90

aag gtg ttc cag gtg cga aag gtg caa ggc acc aac ttg ggc aaa ata   400
Lys Val Phe Gln Val Arg Lys Val Gln Gly Thr Asn Leu Gly Lys Ile
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tat gcc atg aaa gtc cta agg aag gcc aaa att gtg cgc aat gcc aag   448
Tyr Ala Met Lys Val Leu Arg Lys Ala Lys Ile Val Arg Asn Ala Lys
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gac aca gca cac aca cgg gct gag cgg aac att cta gag tca gtg aag   496
Asp Thr Ala His Thr Arg Ala Glu Arg Asn Ile Leu Glu Ser Val Lys
125                 130                 135                 140

cac ccc ttt att gtg gaa ctg gcc tat gcc ttc cag act ggt ggc aaa   544
His Pro Phe Ile Val Glu Leu Ala Tyr Ala Phe Gln Thr Gly Gly Lys
                145                 150                 155

ctc tac ctc atc ctt gag tgc ctc agt ggt ggc gag ctc ttc acg cat   592
Leu Tyr Leu Ile Leu Glu Cys Leu Ser Gly Gly Glu Leu Phe Thr His
            160                 165                 170

ctg gag cga gag ggc atc ttc ctg gaa gat acg gcc tgc ttc tac ctg   640
Leu Glu Arg Glu Gly Ile Phe Leu Glu Asp Thr Ala Cys Phe Tyr Leu
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gct gag atc acg ctg gcc ctg ggc cat ctc cac tcc cag ggc atc atc   688
Ala Glu Ile Thr Leu Ala Leu Gly His Leu His Ser Gln Gly Ile Ile
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tac cgg gac ctc aag ccc gag aac atc atg ctc agc agc cag ggc cac   736
Tyr Arg Asp Leu Lys Pro Glu Asn Ile Met Leu Ser Ser Gln Gly His
205                 210                 215                 220

atc aaa ctg acc gac ttt gga ctc tgc aag gag tct atc cat gag ggc   784
Ile Lys Leu Thr Asp Phe Gly Leu Cys Lys Glu Ser Ile His Glu Gly
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gcc gtc act cac acc ttc tgc ggc acc att gag tac atg gcc cct gag   832
Ala Val Thr His Thr Phe Cys Gly Thr Ile Glu Tyr Met Ala Pro Glu
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att ctg gtg cgc agt ggc cac aac cgg gct gtg gac tgg tgg agc ctg   880
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Gly Ala Leu Met Tyr Asp Met Leu Thr Gly Ser Pro Pro Phe Thr Ala
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gag aac cgg aag aaa acc atg gat aag atc atc agg ggc aag ctg gca   976
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Leu Lys Arg Asn Pro Ser Gln Arg Ile Gly Gly Gly Pro Gly Asp Ala
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gct gat gtg cag aga cat ccc ttt ttc cgg cac atg aat tgg gac gac   1120
Ala Asp Val Gln Arg His Pro Phe Phe Arg His Met Asn Trp Asp Asp
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gag gag gac gtg agc cag ttt gat acc cgc ttc aca cgg cag acg ccg   1216
Glu Glu Asp Val Ser Gln Phe Asp Thr Arg Phe Thr Arg Gln Thr Pro
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gag ggc ttc tcc ttc cag ccc aag ctg cgc tca ccc agg cgc ctc aac   1360
Glu Gly Phe Ser Phe Gln Pro Lys Leu Arg Ser Pro Arg Arg Leu Asn
        415                 420                 425

agt agc ccc cgg gtc ccc gtc agc ccc ctc aag ttc tcc cct ttt gag   1408
Ser Ser Pro Arg Val Pro Val Ser Pro Leu Lys Phe Ser Pro Phe Glu
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Gly Phe Arg Pro Ser Pro Ser Leu Pro Glu Pro Thr Glu Leu Pro Leu
445                 450                 455                 460

cct cca ctc ctg cca ccg ccg ccg ccc tcg acc acc gcc cct ctc ccc   1504
Pro Pro Leu Leu Pro Pro Pro Pro Ser Thr Thr Ala Pro Leu Pro
                465                 470                 475

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Ile Arg Pro Pro Ser Gly Thr Lys Lys Ser Lys Arg Gly Arg Gly Arg
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Pro Gly Arg
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 65                  70                  75                  80

Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly Lys Val Phe Gln
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Val Arg Lys Val Gln Gly Thr Asn Leu Gly Lys Ile Tyr Ala Met Lys
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Val Leu Arg Lys Ala Lys Ile Val Arg Asn Ala Lys Asp Thr Ala His
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Val Glu Leu Ala Tyr Ala Phe Gln Thr Gly Gly Lys Leu Tyr Leu Ile
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Leu Glu Cys Leu Ser Gly Gly Glu Leu Phe Thr His Leu Glu Arg Glu
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Gly Ile Phe Leu Glu Asp Thr Ala Cys Phe Tyr Leu Ala Glu Ile Thr
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Leu Ala Leu Gly His Leu His Ser Gln Gly Ile Ile Tyr Arg Asp Leu
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Lys Pro Glu Asn Ile Met Leu Ser Ser Gln Gly His Ile Lys Leu Thr
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Ser Gly His Asn Arg Ala Val Asp Trp Trp Ser Leu Gly Ala Leu Met
            260                 265                 270

Tyr Asp Met Leu Thr Gly Ser Pro Pro Phe Thr Ala Glu Asn Arg Lys
        275                 280                 285

Lys Thr Met Asp Lys Ile Ile Arg Gly Lys Leu Ala Leu Pro Pro Tyr
    290                 295                 300

Leu Thr Pro Asp Ala Arg Asp Leu Val Lys Lys Phe Leu Lys Arg Asn
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Pro Ser Gln Arg Ile Gly Gly Gly Pro Gly Asp Ala Ala Asp Val Gln
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            340                 345                 350

Arg Val Asp Pro Pro Phe Arg Pro Cys Leu Gln Ser Glu Glu Asp Val
        355                 360                 365

Ser Gln Phe Asp Thr Arg Phe Thr Arg Gln Thr Pro Val Asp Ser Pro
    370                 375                 380

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385                 390                 395                 400

Thr Tyr Val Ala Pro Ser Val Leu Asp Ser Ile Lys Glu Gly Phe Ser
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Phe Gln Pro Lys Leu Arg Ser Pro Arg Arg Leu Asn Ser Ser Pro Arg
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Val Pro Val Ser Pro Leu Lys Phe Ser Pro Phe Glu Gly Phe Arg Pro
        435                 440                 445

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Phe Tyr Pro Ala Pro Asp Phe Arg Asp Arg Glu Ala Glu Asp Met Ala
 10                  15                  20                  25

gga gtg ttt gac ata gac ctg gac cag cca gag gac gcg ggc tct gag   150
Gly Val Phe Asp Ile Asp Leu Asp Gln Pro Glu Asp Ala Gly Ser Glu
                 30                  35                  40

gat gag ctg gag gag ggg ggt cag tta aat gaa agc atg gac cat ggg   198
Asp Glu Leu Glu Glu Gly Gly Gln Leu Asn Glu Ser Met Asp His Gly
             45                  50                  55

gga gtt gga cca tat gaa ctt ggc atg gaa cat tgt gag aaa ttt gaa   246
Gly Val Gly Pro Tyr Glu Leu Gly Met Glu His Cys Glu Lys Phe Glu
         60                  65                  70

atc tca gaa act agt gtg aac aga ggg cca gaa aaa atc aga cca gaa   294
Ile Ser Glu Thr Ser Val Asn Arg Gly Pro Glu Lys Ile Arg Pro Glu
     75                  80                  85

tgt ttt gag cta ctt cgg gta ctt ggt aaa ggg ggc tat gga aag gtt   342
Cys Phe Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly Lys Val
 90                  95                 100                 105

ttt caa gta cga aaa gta aca gga gca aat act ggg aaa ata ttt gcc   390
Phe Gln Val Arg Lys Val Thr Gly Ala Asn Thr Gly Lys Ile Phe Ala
                110                 115                 120

atg aag gtg ctt aaa aag gca atg ata gta aga aat gct aaa gat aca   438
Met Lys Val Leu Lys Lys Ala Met Ile Val Arg Asn Ala Lys Asp Thr
            125                 130                 135

gct cat aca aaa gca gaa cgg aat att ctg gag gaa gta aag cat ccc   486
Ala His Thr Lys Ala Glu Arg Asn Ile Leu Glu Glu Val Lys His Pro
        140                 145                 150

ttc atc gtg gat tta att tat gcc ttt cag act ggt gga aaa ctc tac   534
Phe Ile Val Asp Leu Ile Tyr Ala Phe Gln Thr Gly Gly Lys Leu Tyr
    155                 160                 165

ctc atc ctt gag tat ctc agt gga gga gaa cta ttt atg cag tta gaa   582
Leu Ile Leu Glu Tyr Leu Ser Gly Gly Glu Leu Phe Met Gln Leu Glu
170                 175                 180                 185

aga gag gga ata ttt atg gaa gac act gcc tgc ttt tac ttg gca gaa   630
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gac ctg aag ccg gag aat atc atg ctt aat cac caa ggt cat gtg aaa   726
Asp Leu Lys Pro Glu Asn Ile Met Leu Asn His Gln Gly His Val Lys
        220                 225                 230

cta aca gac ttt gga cta tgc aaa gaa tct att cat gat gga aca gtc   774
Leu Thr Asp Phe Gly Leu Cys Lys Glu Ser Ile His Asp Gly Thr Val
    235                 240                 245

aca cac aca ttt tgt gga aca ata gaa tac atg gcc cct gaa atc ttg   822
Thr His Thr Phe Cys Gly Thr Ile Glu Tyr Met Ala Pro Glu Ile Leu
250                 255                 260                 265

atg aga agt ggc cac aat cgt gct gtg gat tgg tgg agt ttg gga gca   870
Met Arg Ser Gly His Asn Arg Ala Val Asp Trp Trp Ser Leu Gly Ala
                270                 275                 280

tta atg tat gac atg ctg act gga gca ccc cca ttc act ggg gag aat   918
Leu Met Tyr Asp Met Leu Thr Gly Ala Pro Pro Phe Thr Gly Glu Asn
            285                 290                 295

aga aag aaa aca att gac aaa atc ctc aaa tgt aaa ctc aat ttg cct   966
Arg Lys Lys Thr Ile Asp Lys Ile Leu Lys Cys Lys Leu Asn Leu Pro
        300                 305                 310

ccc tac ctc aca caa gaa gcc aga gat ctg ctt aaa aag ctg ctg aaa   1014
Pro Tyr Leu Thr Gln Glu Ala Arg Asp Leu Leu Lys Lys Leu Leu Lys
    315                 320                 325

aga aat gct gct tct cgt ctg gga gct ggt cct ggg gac gct gga gaa   1062
Arg Asn Ala Ala Ser Arg Leu Gly Ala Gly Pro Gly Asp Ala Gly Glu
330                 335                 340                 345

gtt caa gct cat cca ttc ttt aga cac att aac tgg gaa gaa ctt ctg   1110
Val Gln Ala His Pro Phe Phe Arg His Ile Asn Trp Glu Glu Leu Leu
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gct cga aag gtg gag ccc ccc ttt aaa cct ctg ttg caa tct gaa gag   1158
Ala Arg Lys Val Glu Pro Pro Phe Lys Pro Leu Leu Gln Ser Glu Glu
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gat gta agt cag ttt gat tcc aag ttt aca cgt cag aca cct gtc gac   1206
Asp Val Ser Gln Phe Asp Ser Lys Phe Thr Arg Gln Thr Pro Val Asp
        380                 385                 390

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Ser Pro Asp Asp Ser Thr Leu Ser Glu Ser Ala Asn Gln Val Phe Leu
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Pro Arg Thr Pro Val Ser Pro Val Lys Phe Ser Pro Gly Asp Phe Trp
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Gly Arg Gly Ala Ser Ala Ser Thr Ala Asn Pro Gln Thr Pro Val Glu
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Tyr Pro Met Glu Thr Ser Gly Ile Glu Gln Met Asp Val Thr Met Ser
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Gly Glu Ala Ser Ala Pro Leu Pro Ile Arg Gln Pro Asn Ser Gly Pro
490                 495                 500                 505

tac aaa aaa caa gct ttt ccc atg atc tcc aaa cgg cca gag cac ctg   1590
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            525

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         35                  40                  45

Gln Leu Asn Glu Ser Met Asp His Gly Gly Val Gly Pro Tyr Glu Leu
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Gly Met Glu His Cys Glu Lys Phe Glu Ile Ser Glu Thr Ser Val Asn
 65                  70                  75                  80

Arg Gly Pro Glu Lys Ile Arg Pro Glu Cys Phe Glu Leu Leu Arg Val
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Leu Gly Lys Gly Gly Tyr Gly Lys Val Phe Gln Val Arg Lys Val Thr
            100                 105                 110

Gly Ala Asn Thr Gly Lys Ile Phe Ala Met Lys Val Leu Lys Lys Ala
        115                 120                 125

Met Ile Val Arg Asn Ala Lys Asp Thr Ala His Thr Lys Ala Glu Arg
    130                 135                 140

Asn Ile Leu Glu Glu Val Lys His Pro Phe Ile Val Asp Leu Ile Tyr
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Ala Phe Gln Thr Gly Gly Lys Leu Tyr Leu Ile Leu Glu Tyr Leu Ser
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Gly Gly Glu Leu Phe Met Gln Leu Glu Arg Glu Gly Ile Phe Met Glu
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    290                 295                 300

Ile Leu Lys Cys Lys Leu Asn Leu Pro Pro Tyr Leu Thr Gln Glu Ala
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Phe Lys Pro Leu Leu Gln Ser Glu Glu Asp Val Ser Gln Phe Asp Ser
    370                 375                 380

Lys Phe Thr Arg Gln Thr Pro Val Asp Ser Pro Asp Asp Ser Thr Leu
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Ser Glu Ser Ala Asn Gln Val Phe Leu Gly Phe Thr Tyr Val Ala Pro
                405                 410                 415

Ser Val Leu Glu Ser Val Lys Glu Lys Phe Ser Phe Glu Pro Lys Ile
            420                 425                 430

Arg Ser Pro Arg Arg Phe Ile Gly Ser Pro Arg Thr Pro Val Ser Pro
        435                 440                 445

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Ser Gln Lys

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1 5 10 15

Gln Lys

INTERNATIONAL S CH REPORT

[redacted]onal Application No
PCT/US 99/17595

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/62 C12N9/12 C12N5/10 C07K16/40
G01N33/50 G01N33/566 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GOUT I. ET AL.: "Molecular cloning and characterization of a novel p70 S6 kinase beta containing a proline-rich region" J. BIOL. CHEM., vol. 273, no. 46, 13 November 1998 (1998-11-13), pages 30061-30064, XP002124654 the whole document	1-36
P,X	SAITOH M. ET AL.: "Cloning and characterization of p70(S6 beta) defines a novel family of p70 S6 kinases" BIOCHEM. BIOPHYS. RES. COMMUN., vol. 253, no. 2, 18 December 1998 (1998-12-18), pages 471-476, XP002124655 the whole document	1-36

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1999	11/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 24463 A (INCYTE PHARMA INC ;MATHUR PREETE (US); REDDY ROOPA (US); AU YOUNG) 20 May 1999 (1999-05-20) compare nt 180-1450 of seq. ID 10 and nt 101-1370 of seq. ID 1 of present application	1-4
A	WO 98 18935 A (CIBA GEIGY AG ;THOMAS GEORGE (FR); KOZMA SARA (FR)) 7 May 1998 (1998-05-07) abstract claims 1-11	1-36
A	WO 93 19752 A (DANA FARBER CANCER INST INC) 14 October 1993 (1993-10-14) abstract	1-36
A	MUKHOPADHYAY N.K. ET AL.: "An array of insulin-activated, proline-directed serine/threonine kinases phosphorylate the p70 S6 kinase" J. BIOL. CHEM., vol. 267, no. 5, 15 February 1995 (1995-02-15), pages 3325-3335, XP002124656 the whole document	1-36
A	WENG Q.P. ET AL.: "Regulation of the p70 S6 kinase by phosphorylation in vivo" J. BIOL. CHEM., vol. 273, no. 26, 26 June 1998 (1998-06-26), pages 16621-16629, XP002124657 the whole document	1-36
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passagee	Relevant to claim No.
A	HAN J -W ET AL: "RAPAMYCIN, WORTMANNIN, AND THE METHYLXANTHINE SQ20006 INACTIVATE P70S6K BY INDUCING DEPHOSPHORYLATION OF THE SAME SUBSET OF SITES" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 270, no. 36, page 21396-21403 XP002057557 ISSN: 0021-9258 the whole document	1-36
A	PEARSON R B ET AL: "THE PRINCIPAL TARGET OF RAPAMYCIN-INDUCED P70S6K INACTIVATION IS A NOVEL PHOSPHORYLATION SITE WITHIN A CONSERVED HYDROPHOBIC DOMAIN" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 14, no. 21, page 5279-5287 XP000561164 ISSN: 0261-4189 the whole document	1-36
A	DENNIS P B ET AL: "THE PRINCIPAL RAPAMYCIN-SENSITIVE P70S6K PHOSPHORYLATION SITES, T-229 AND T-389, ARE DIFFERENTIALLY REGULATED BY RAPAMYCIN-INSENSITIVE KINASE KINASES" MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 16, no. 11, page 6242-6251 XP002057559 ISSN: 0270-7306 the whole document	1-36
A	WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA () 29 January 1998 (1998-01-29) abstract claims 1-9	1-36
A	PROUD C G: "P70 S6 KINASE: AN ENIGMA WITH VARIATIONS" TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 21, no. 5, page 181-185 XP002057556 ISSN: 0968-0004 the whole document	1-36

INTERNATIONAL SEARCH REPORT

.ernational application No.
PCT/US 99/17595

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 10-11, as far as methods in vivo are concerned are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims 10-11 refer to modulating compounds, and claims 25-26 refer to binding partners of p70 S6K (beta) without giving a true technical characterization. Moreover, no such compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/17595

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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